

IMPROVED METHOD FOR THE PREVENTION OR REDUCTION OF HAZE IN  
BEVERAGES

5       The invention relates to a method for the prevention or reduction of  
haze in a beverage by the addition of an endoprotease and to new beverages  
obtainable by the method according to the invention. It also relates to new  
endoproteases.

10       Haze is a well-known phenomenon in the beverage industry. Haze  
can for example be present in beer, wine and fruit juice. Haze formation can occur at  
different stages during the brewing process. In "Enzymes in food processing" edited by  
T. Nagodawithana and G. Reed, 3<sup>rd</sup> edition, Academic press Inc., San Diego, Chapter  
V, p.448-449, it has been proposed that the haze in beer is the result of interactions  
between beer proteins and polyphenolic procyanidins. It is explained that in beer haze  
is often formed upon chilling of the beer. Beer is fermented and then matured, often  
15       under chilled conditions. To achieve clarity, beer is often filtered while cold. In spite of  
the filtration, beer often becomes cloudy after it is packaged and distributed to  
customers and chilled again before serving. Eventually haze is even formed in beer  
when it is not or no longer chilled and sediment may develop. Haze formation is  
undesirable because the cloudiness caused by haze formation resembles cloudiness  
20       produced by microbial spoilage, which is undesirable, especially for bright beers.

      In "Industrial Enzymology", 2<sup>nd</sup> edition, Chapter 2.6, p.124-125, it has  
been described that haze in beer can result from the cross-linkage of the high  
molecular weight hordein fraction of malt, containing a high proportion of hydrophobic  
amino acids, which combines with polyphenols principally consisting of  
25       proanthocyanidins and catechins (flavanoids). It is described that small amounts of  
carbohydrates and trace mineral ions are also involved in haze formation, as well as  
oxidation, which is stated to play an important part in polymerization of polyphenols to  
produce irreversible haze. It is proposed that polyphenols combine slowly with protein  
to form chill haze when cooled, but which redissolve when warmed up. Eventually,  
30       however, as polyphenols polymerize and increase in size they become insoluble at  
room temperature to form irreversible or permanent haze.

      In several other publications it has been proposed that the formation  
of haze in for example beer, wine and fruit juice, coffees and teas is the result of  
interactions between proteins and polyphenols (K.J. Siebert et al, J. Agric. Food Chem.  
35       44 (1996) 1997-2005 and K.J. Siebert et al, J. Agric. Food Chem. 44 (1996) 80-85, K.J.  
Siebert, J. Agric. Food Chem. 47 (1999) 353-362). Since its discovery by L.

Wallerstein in 1911, it has been known that a method for the reduction of chill haze formation in beer is the addition of papain to the beer. Papain is an extract of papaya having proteolytic activity. In "Enzymes in food processing" edited by T. Nagodawithana and G. Reed, 3<sup>rd</sup> edition, Academic press Inc., San Diego, Chapter V, p.448-449 papain is described as being far superior to any other enzyme for the prevention of chill haze in beer. The exact mechanism by which papain works, however, has never been determined ("Enzymes in food processing" edited by T. Nagodawithana and G. Reed, 3<sup>rd</sup> edition, Academic press Inc., San Diego, Chapter V, p.448-449).

A disadvantage of the use of papain however, is that it has a negative effect on foam. Proteins are necessary to form stable foam on beer. By its proteolytic activity, however, papain adversely affects head foam stability.

Haze formation in wine has been discussed in e.g. "Enzymes in food processing" edited by T. Nagodawithana and G. Reed, 3<sup>rd</sup> edition, Academic press Inc., San Diego, Chapter 16, p.425, where it is described that grape proteins are held responsible for the formation of haze during the storage of wine. If precipitation is formed in wine after bottling, the wine becomes less attractive to the consumer, which will affect sales. To prevent precipitation, for example bentonite is used. Although bentonite and other adsorbents are successful in removing the proteins, it is not selective and removes other desirable compounds from wine, often affecting the organoleptic properties of wine. In addition, the use of bentonite results in a considerable loss of wine and the dumping of waste containing bentonite presents difficulties.

Although the mechanism is not well understood, it is assumed that the addition of papain hydrolyses the protein in beer to such an extent that a protein-polyphenol haze is not formed or is formed to a smaller extent. Bentonite is used for a similar reason in wine: by absorbing proteins it prevents the formation of protein-polyphenol haze and precipitates. Instead of removing the protein, however, polyphenols may be removed to reduce or prevent haze formation. A typical example of a compound used to remove polyphenols from beverages is polyvinylpolypyrrolidone (PVPP). Lately it has been recognized that polyphenols are important anti-oxidants. Because of all the beneficial effects attributed to anti-oxidants, the option of removing polyphenols from beverages is not the most attractive way to prevent the formation of haze.

Since all known techniques for the prevention or removal of haze have drawbacks, there is still a need for a new method for the prevention or reduction

of haze in beverages.

It is an object of the present invention to provide a method for the prevention or reduction of haze in a beverage.

Surprisingly, it has been found that this object is achieved by providing a method for the prevention or reduction of haze in a beverage wherein an endoprotease with an acidic pH optimum is added to the beverage. Preferably this endoprotease is an isolated endoprotease that has prolyl-specific endoprotease activity and/or hydroxyprolyl-specific activity and/or alanine specific activity.

In the framework of this invention the term "beverage" includes beverages in all stages of their preparation. Thus, a beverage is not only a beverage ready for consumption but also any composition used to prepare the beverage. For example, wort as used in beer preparation is encompassed by the term "beverage" as used herein. Also, the addition of a prolyl-specific endoprotease during the preparation of a beverage to compositions that are not or not entirely liquid is intended to fall within the method according to the invention. A prolyl-specific endoprotease added to a mash at the start of beer brewing is an example of such a composition.

A prolyl specific endoprotease (Endo-Pro) is defined as a preferably purified endoprotease that cuts proteins or peptides near or at places where the protein or peptide contains a prolyl-residue in its chain. Preferably, in a method according to the invention, a prolyl specific endoprotease is used that cuts proteins or peptides at places where the protein or peptide contains a prolyl-residue.

The terms Endo-Pro, prolyl-specific endoprotease, proline-specific endoprotease, proline-specific endopeptidase and peptide having a prolyl-specific activity or similar expressions are used interchangeably.

A hydroxy-prolyl specific endoprotease (Endo-Hydroxy-Pro) is defined as a preferably purified endoprotease that cuts proteins or peptides near or at places where the protein or peptide contains a hydroxy-prolyl-residue in its chain. Preferably, in a method according to the invention, a hydroxy-prolyl specific endoprotease is used that cuts proteins or peptides at places where the protein or peptide contains a hydroxy-prolyl-residue.

An alanine specific endoprotease (Endo-Ala) is defined as a preferably purified endoprotease that cuts proteins or peptides near or at places where the protein or peptide contains an alanine-residue in its chain. Preferably, in a method according to the invention, an alanine-specific endoprotease is used that cuts proteins or peptides at places where the protein or peptide contains an alanine-residue.

Endoproteases having a prolyl-specific activity are known (E.C.

3.4.21.26). However, the use of prolyl-specific endoproteases or even hydroxy-prolyl specific endoproteases or alanine specific endoproteases for the prevention or reduction of haze in beverages has never been described or suggested.

The words peptide and protein are used interchangeably herein.

5 Also, the words "haze", "cloudiness" and "turbidity" are used interchangeably.

In an even more preferred method according to the invention, a prolyl-specific endoprotease is used that cuts prolyl-residues at their C-terminus. A prolyl-specific endoprotease that cuts prolyl-residues at their NH<sub>2</sub>-terminus is for example described in a publication in Nature of 15 January 1998, Vol.391, p.301-304.

10 Further haze reduction in a beverage may be achieved by treatment with a prolyl specific and/or hydroxyprolyl specific and/or alanine specific endoprotease in combination with an auxiliary enzyme.

Under certain conditions and in certain beverages, it may be that a further reduction of haze is desired than the level of haze reduction that is achievable with an Endo-Pro, Endo-Hydroxy-Pro and/or Endo-Ala treatment alone. In such cases it may be beneficial to add an auxiliary proteolytic enzyme before, during or after the Endo-Pro, Endo-Hydroxy-Pro and/or Endo-Ala treatment. Such auxiliary enzymes may be endoproteases or exoproteases such as tripeptidylpeptidases and/or carboxypeptidases and/or peptidyl-dipeptidases.

20 Object of this additional treatment is to further increase the solubility of the peptides that remain after Endo-Pro, Endo-Hydroxy-Pro and/or Endo-Ala treatment and/or to further reduce the interaction of the remaining polypeptides with polyphenols.

This objective is met in that an exoproteases such as tripeptidylpeptidase and/or carboxypeptidase and/or peptidyl-dipeptidases is added to the beverage that is able to remove the carboxy-terminal proline residues from the peptides that remain after Endo-Pro treatment. Alternatively, or in combination with such an exopeptidase, the peptides that remain after Endo-Pro treatment may be further solubilised by treatment with an endoprotease. Especially suited for that purpose are enzymes that are able to cleave peptide bonds at either the N- or C-terminal position of glycine, alanine, serine, asparagine and glutamine residues.

Carboxypeptidases that have activity towards synthetic chromogenic peptides FA-Pro or FA Pro-Pro were found to very useful as auxiliary enzymes

35 Auxiliary Endo-Proteolytic enzymes with specificities as mentioned above may be commercially obtained or may alternatively be selected with methods known in the art, for instance with the aid of synthetic chromogenic peptides such as

## Z-A-A-A-pNA

wherein

- 5    pNA                = paranitroanilide,  
     Z                = benzyloxycarbonyl,  
     A                = amino acid glycine, alanine, serine, asparagines or glutamine

10                    Auxillary peptidyl-dipeptidases that have activity towards synthetic  
chromogenic peptides such as FA-Leu-Pro or FA-Phe-Pro wherein FA= furylacryloyl  
were found to be very useful.

                    The treatment with auxiliary enzymes should preferably have no  
adverse effect on the taste, texture or mouth-feel of the beverage. It is preferred that  
these auxiliary enzymes have an acidic pH optimum or are active under acidic  
15    conditions, preferably below, at or around pH 6.0, 5.0, 4.5 or 4.0 or even more  
preferred below, at or around pH 3.0.

                    In the production of most beers, special attention is required when  
adding (auxiliary) proteolytic enzymes in order not to destroy the ability of the beer to  
form foam.

20                    In a conventional beer brewing process, cereals are milled and  
mashed and the resulting mash is filtered to give the wort. The wort is then boiled to  
inactivate all residual enzymic activities and subsequently used to support yeast  
growth. After yeast growth and filtering, the beer is lagered. It has been described that  
in this lagering stage, papain (collupuline) may be added to the beverage in order to  
25    prevent haze formation. However, the papaine destroys the foam forming ability of the  
beer.

                    Responsible for beer foam is a protein called LTP1 (Lipid Transfer  
Protein), a 10 kDa barley protein with a very sturdy 3-dimensional structure so that  
proteases normally present during the mashing stage cannot hydrolyse this molecule.  
30    However, during the wort boiling stage, LTP1 unfolds so that it becomes susceptible to  
enzymatic cleavage. To be active in foam formation, the LTP1 needs a certain  
minimum size. It is now thought that incubation with Collupuline during lagering  
(i.e. after wort boiling) destroys LTP1 and thereby the foam forming ability of the beer.

                    The use of an acidic endoprotease, in particular Endo-Pro, Endo-  
35    Hydroxy-Pro and/or Endo-Ala during either the mashing or the lagering stage reduces  
haze formation, but does not destroy or seriously affect LTP1 and therefore the use of

such enzymes has no negative effects on foam formation.

Moreover, the removal of a carboxy-terminal proline residue from the peptides that resulted from the digestion with Endo-Pro, resulted in an additional reduction of haze in beer. Remarkably good results in this respect were obtained when  
5 a proline-specific carboxypeptidase isolated from *Xanthomonas* was used in combination with Endo-Pro. Also, peptidyl-dipeptidase A (EC 3.4.15.1) was successfully used to that effect.

Milk-clotting enzymes like Fromase® are also very well suited as auxiliary enzyme in the reduction or prevention of haze when used in combination with Endo-  
10 Pro. Fromase® is well suited for this purpose since it does not affect LPT1 and therewith preserves the foam forming ability of the beer.

Fromase is a commercial product (DSM Food Specialities) obtained from *Rhizomucor miehei* that is used in cheese production. Fromase is a so-called aspartic protease (EC 3.4.23). These enzymes are characterised by very low pH  
15 optima and an outspoken preference for cleaving peptide bonds between bulky, hydrophobic amino acid residues such as Phe-Phe, Phe-Tyr and Leu-Tyr. Other aspartic acid proteases are pepsin, cathepsin and the various acid proteases from different fungi.

Further haze reduction in wine may also be achieved by additional  
20 treatment with auxiliary enzymes in combination with Endo-Pro, Endo-Hydroxy-Pro or Endo-Ala hydrolysis.

A protein called chitinase is known to be the main cause of haze problems in wine. This chitinase originates from the grape and is rich in glycine, alanine and serine residues. Since wine has a very low pH, we tried to hydrolyse this chitinase  
25 with a complex mixture of acidic endoproteases. Addition of high concentrations of a product called AP 50.000 from Shin Nihon, Japan had no effect on haze formation in wine. This finding corresponds with the conclusions of Ferreira *et al.* (Trends in Food Science & Technology 12 (2002) 230-239 stating that all strategies based on the proteolysis of wine proteins have proved unsuccessful in practice and are probably  
30 futile.

Incubation of wine (white and red) with an enzyme preparation according to the invention is described in Example 7. A further reduction of the haze in wine could be obtained by using auxiliary enzymes such as an exopeptidase that removed the carboxy-terminal proline residues and/or an endoprotease with specificity  
35 for glycine.

An additional advantage of using Endo-Pro in wine (or any other

beverage with a very low pH) is that the Endo-Pro used in the experiments of Example 7 is also capable of cleaving peptide bonds after alanine residues as well as at hydroxy-proline residues under acidic conditions (Example 13).

To quantify the amount of haze in a beverage, a turbidimeter is often used. In a turbidimeter the amount of light is measured that is scattered at a prediscrined angle relative to the direction of the incident light beam. Turbidity measurements are very suitable for the measurement of haze formed as the result of protein-polyphenol interactions.

A polyphenol is defined as a compound having a chemical structure which contains at least two aromatic rings substituted with at least one hydroxyl group or having a chemical structure which contains at least one aromatic ring substituted with at least two hydroxyl groups.

Examples of polyphenols are tannins and flavonoids, which include for example catechins, flavonols and anthocyanins.

As is typical for enzyme activities, the activity of prolyl-specific endoproteases is dependent on the pH. In a preferred embodiment of the method according to the invention, an endoprotease is added to the beverage having a maximum prolyl specific activity at a pH that corresponds to the pH of the beverage it is added to. Preferred beverages are beverages containing proteins. In another preferred embodiment, the beverage contains proteins and polyphenols. Preferred beverages are beverages having a pH value below 7.

The method according to the invention is advantageously applied to beer, wine and fruit juice. It may also advantageously be applied to alcoholic beverages other than beer and wine.

The term "beer" as used herein is intended to cover at least beer prepared from mashes prepared from unmalted cereals as well as all mashes prepared from malted cereals, and all mashes prepared from a mixture of malted and unmalted cereals. The term "beer" also covers beers prepared with adjuncts, and beers with all possible alcohol contents.

Fruit juice may be juice obtained from for example red berries, strawberries, apples, pears, tomatoes, citrus fruits, vegetables etc.

The amount of proline-specific endoprotease that is added to a beverage in the method according to the invention may vary between wide limits. In a preferred embodiment of the method according to the invention at least 150 milli-units of proline-specific endoprotease activity, whereby the activity was determined by an activity measurements using Z-Gly-Pro-pNA as a substrate, per gram protein in the

beverage is added.

More preferably, at least 500 milli-units of proline-specific endoprotease is added to the beverage, and most preferably, at least 1 unit of proline-specific endoprotease is added.

5                   A maximum amount of proline-specific endoprotease activity to be added cannot be specified. The maximum amount is for example dependent on the desired amount of haze reduction or prevention, the composition of the beverage, the pH of the beverage and the pH at which the endoprotease has its maximum activity.

10                   A prolyl-specific endoprotease may be added at different stages during the preparation of a beverage.

                  During the preparation process of beer, the prolyl-specific endoprotease is advantageously added to a mash. In another embodiment, the prolyl-specific endoprotease Endo-Pro is added to a fermented beer. The prolyl-specific endoprotease may advantageously be added to the mashing or maturation step in a  
15                   process for the preparation of beer. Most preferred is the addition of the enzyme to the wort after the boiling step.

                  During the preparation of wine, the prolyl-specific endoprotease is preferably added to a fermented wine. The prolyl-specific endoprotease may advantageously be added after alcoholic fermentation or after malolactic fermentation  
20                   in a process for the production of a wine. However, the enzyme may also be added to the clear grape juice, i.e. before the alcoholic fermentation.

                  In a process for the preparation of a fruit juice, the prolyl-specific endoprotease is preferably added during the maceration or depectinization.

25                   Since haze formation often occurs in acidic beverages such as for example beer, wine and fruit juice, prolyl-specific endoproteases having a prolyl specific activity at a pH value below 7 are preferably used. More preferable, prolyl-specific endoproteases having a maximum prolyl specific activity at a pH value below 7.0, or 6.0 are used in the method according to the invention. Most preferred are endoproteases with a pH optimum at or below pH 5.0.

30                   The present invention provides such a peptide, having proline-specific endoprotease activity with a pH optimum around pH 5.0, or between 4.0 and 5.0.

                  The present invention further provides an isolated polypeptide that has proline-specific endoprotease activity selected from the group consisting of:



- (a) a polypeptide which has an amino acid sequence which has at least 40% overall amino acid sequence identity with SEQ ID NO: 4, SEQ ID NO: 5, OR SEQ ID NO: 7 or a fragment thereof;
- (b) a polypeptide which is encoded by a polynucleotide which hybridizes with
- 5 (i) the nucleic acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6 or a fragment thereof which is at least 80% or 90% identical over 60, preferably over 100 nucleotides, more preferably at least 90% identical over 200 nucleotides, or (ii) a nucleic acid sequence complementary to the nucleic acid sequence of (i)

10 It also provides a nucleic acid molecule encoding the prolyl-specific endoprotease Endo-Pro.

The invention also relates to purified or isolated polypeptides having prolyl-specific endoprotease activity. Preferred are purified prolyl-specific

15 endoproteases having a maximum activity at pH values below 7.

The invention provides an isolated polypeptide having the amino acid sequence according to SEQ ID NO: 4, SEQ ID NO: 5 or SEQ ID NO: 7, or an amino acid sequence obtainable by expressing the polynucleotide sequences of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, OR SEQ ID NO: 6 in an appropriate host. Also, a

20 peptide or polypeptide comprising a functional equivalent of the above polypeptides is comprised within the present invention. The above polypeptides are collectively comprised in the term "polypeptides according to the invention"

The terms "peptide" and "oligopeptide" are considered synonymous (as is commonly recognized) and each term can be used interchangeably as the

25 context requires to indicate a chain of at least two amino acids coupled by peptidyl linkages. The word "polypeptide" is used herein for chains containing more than seven amino acid residues. All oligopeptide and polypeptide formulas or sequences herein are written from left to right and in the direction from amino terminus to carboxy terminus. The one-letter code of amino acids used herein is commonly known in the art

30 and can be found in Sambrook, et al. (Molecular Cloning: A Laboratory Manual, 2<sup>nd</sup>, ed. Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989)

By "isolated" or "purified" polypeptide or protein is intended a polypeptide or protein removed from its native environment. For example,

35 recombinantly produced polypeptides and proteins expressed in host cells are considered isolated for the purpose of the invention as are native or recombinant

polypeptides which have been substantially purified by any suitable technique such as, for example, a simple ultrafiltration step to separate the enzyme from the cell mass or the single-step purification method disclosed in Smith and Johnson, Gene 67:31-40 (1988).

5                   The polypeptide according to the invention can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin  
10 chromatography. Most preferably, high performance liquid chromatography ("HPLC") is employed for purification.

                  Polypeptides of the present invention include naturally purified products, products of chemical synthetic procedures, and products produced by recombinant techniques from a prokaryotic or eukaryotic host, including, for example,  
15 bacterial, yeast, higher plant, insect and mammalian cells. Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. In addition, polypeptides of the invention may also include an initial modified methionine residue, in some cases as a result of host-mediated processes.

20                   An advantageous embodiment of the invention concerns a purified or isolated polypeptide having Endo-Pro activity. Such purified or isolated polypeptide may be obtained from a fermentation broth wherein an organism according to the invention, such as an *A. niger* strain carrying a polynucleotide according to the invention, has been cultured. A person skilled in the art will know how to obtain at least  
25 partially purified enzyme from the supernatant of such a culture. In its most basic form the producing cells are separated from the fermentation broth by centrifugation. The resulting liquid is then filtered using a filter-aid, followed by an ultra-filtration step so that an enzyme solution containing 1 to 50 enzyme units per millilitre is obtained. Under ideal conditions the enzyme can be used as such, i.e. without additional purification. If  
30 required the shelf stability of the enzyme can be improved by spray drying or stabilizing the enzyme by lowering the water activity of the enzyme solution e.g. by adding a polyol and a preservative.

                  A particularly advantageous method of purification is the following. After culturing the cells in an appropriate fermentation broth, the cells were separated  
35 from the culture supernatant by centrifugation. The supernatant had a hazy look. Larger particles remaining in the supernatant were then subsequently removed by

filtration with 0,5% Dicalite or preferably 1.0% Dicalite in order to prevent clogging of the filter that is applied in the next step. Next, Germ reduction filtration was applied to decrease the amount of germs in the solution. Still, the filtrate was not clear. A Millipore filter with a Molecular weight cut-off value of 10kDalton was subsequently used for further reduction of water, salt and sugar content of the solution. A pressure of 1 bar was applied over the filter. Typical yields obtained were between 50 and 92 % based on units present in the fermentation broth versus units obtained in the purified ultrafiltrate. Typical concentrations of enzyme in the ultrafiltrate result in an prolyl-specific endoprotease activity in the range of 4 to 10 Units per ml.

Further purification was obtained by applying either of the following methods:

Lab-scale purification was performed using the Akta Explorer on a 24 ml Q-sepharose FF column (bed height 12 cm / diameter 1.6 cm). 10 ml UF-concentrate was diluted 10 times in buffer A and applied to the column. Proteins were eluted in a gradient: 0 to 50 % B in 20 CV. Buffer A was 20 mM NaAc pH 5.1. Buffer B was 20 mM NaAc + 1 M NaCl pH 5.1. Flow was 5 ml/min.

Purification was performed using the Akta purifier according to work instruction W-0894.A on a 500 ml Q-sepharose FF column (bed height 23.5 cm / diameter 5 cm). 200 ml UF-concentrate was diluted 10 times in buffer A and applied to the column. Proteins were eluted in a gradient: 0 to 40 % B in 20 CV. Buffer A was 20 mM NaAc pH 5.1. Buffer B was 20 mM NaAc + 1 M NaCl pH 5.1. Flow was 10ml/min. Fractions were manually collected.

The obtained product exhibited a single peak on HPSEC and appeared as a single band in SDS PAGE and IEF. It may thus be concluded that prolyl-specific endoprotease may be purified to homogeneity using Q-sepharose FF. Estimated purity was over 90% and specific activity on Z-gly-Pro-pNA was at least 0.094U/mg.

Polypeptides of the invention may be in an isolated form. It will be understood that the polypeptide may be mixed with carriers or diluents which will not interfere with the intended purpose of the polypeptide and still be regarded as isolated. A polypeptide of the invention may also be in a more substantially purified form, in which case it will generally comprise the polypeptide in a preparation in which more than 70%, e.g. more than 80%, 90%, 95%, 98% or 99% of the proteins in the preparation is a polypeptide of the invention.

Polypeptides of the invention may be provided in a form such that they are outside their natural cellular environment. Thus, they may be substantially

isolated or purified, as discussed above, or in a cell in which they do not occur in nature, for example a cell of other fungal species, animals, plants or bacteria.

Advantageously, isolated or purified prolyl-specific endoprotease are used in the method according to the invention.

5 An isolated or purified proline-specific endoprotease according to the invention preferably has at least 10 units of proline specific endoprotease activity per gram of proteinaceous material. These units should be measured using the synthetic peptide Z-Gly-Pro-pNA at 37 degrees C and pH 5, as described in the Methods section.

10 Proline-specific endoproteases are widely found in animals and plants, but their presence in microorganisms appears to be limited. To date, proline-specific endoprotease have been identified in species of *Aspergillus* (EP 0 522 428), *Flavobacterium* (EP 0 967 285) and *Aeromonas* (J.Biochem.113, 790-796), *Xanthomonas* and *Bacteroides*. In contrast to the proline-specific enzymes from most  
15 of these organisms which are active around pH 8, the enzymes according to the invention are optimally active at acidic pH, some even have a pH optimum around pH 5 or below. The proline-specific endoproteases of the invention may be isolated from one of the above-mentioned microbial species, particularly from a species of *Aspergillus*. Preferably, the acidic proline-specific endoprotease Endo-Pro is isolated from a strain  
20 of *Aspergillus niger*. More preferably, the proline-specific endoprotease is isolated from an *Aspergillus niger* host engineered to overexpress a gene encoding a proline-specific endoprotease, although other hosts, such as *E. coli* are suitable expression vectors. For example, the cloning and overproduction of the *Flavobacterium* derived proline-specific endoprotease in, amongst others, *E.coli* has made certain proline-specific  
25 endoproteases available in a pure form. An example of such an overproducing construct is provided in the World Journal of Microbiology & Biotechnology, Vol 11, pp 209-212. An *Aspergillus niger* host is preferably used to produce a non-recombinant self-construct utilizing *A. niger* promoters to drive the expression of a gene encoding an *A. niger* proline-specific endoprotease.

30 In a first embodiment, the present invention provides an isolated polypeptide having an amino acid sequence which has an overall degree of amino acid sequence identity to amino acids of SEQ ID NO: 4, SEQ ID NO: 5, OR SEQ ID NO: 7 (i.e. the polypeptide) of at least about 40%, preferably at least about 50%, preferably at least about 60%, preferably at least about 65%, preferably at least about 70%, more  
35 preferably at least about 80%, even more preferably at least about 90%, still more preferably at least about 95%, and most preferably at least about 97%, and which has

proline specific endoprotease activity.

For the purposes of the present invention, the degree of identity between two or more amino acid sequences is determined by BLAST P protein database search program (Altschul et al., 1997, Nucleic Acids Research 25: 3389-3402) with matrix Blosum 62 and an expected threshold of 10.

A polypeptide of the invention may comprise the amino acid sequence set forth in SEQ ID NO: 4, SEQ ID NO: 5, OR SEQ ID NO: 7 or a substantially homologous sequence, or a fragment of either sequence having proline specific endoprotease activity. In general, the naturally occurring amino acid sequence shown in SEQ ID NO: 4, SEQ ID NO: 5, OR SEQ ID NO: 7 is preferred.

The polypeptide of the invention may also comprise a naturally occurring variant or species homologue of the polypeptide of SEQ ID NO: 4, SEQ ID NO: 5, OR SEQ ID NO: 7.

A variant is a polypeptide that occurs naturally in, for example, fungal, bacterial, yeast or plant cells, the variant having proline specific endoprotease activity and a sequence substantially similar to the protein of SEQ ID NO: 4, SEQ ID NO: 5, OR SEQ ID NO: 7. The term "variants" refers to polypeptides which have the same essential character or basic biological functionality as the proline specific endoprotease of SEQ ID NO: 4, SEQ ID NO: 5, OR SEQ ID NO: 7, and includes allelic variants. Preferably, a variant polypeptide has at least the same level of proline specific endoprotease activity as the polypeptide of SEQ ID NO: 4, SEQ ID NO: 5, OR SEQ ID NO: 7. Variants include allelic variants either from the same strain as the polypeptide of SEQ ID NO: 4, SEQ ID NO: 5, OR SEQ ID NO: 7. or from a different strain of the same genus or species.

Similarly, a species homologue of the inventive protein is an equivalent protein of similar sequence which is an proline specific endoprotease and occurs naturally in another species of *Aspergillus*.

Variants and species homologues can be isolated using the procedures described herein which were used to isolate the polypeptide of SEQ ID NO: 4, SEQ ID NO: 5, OR SEQ ID NO: 7 and performing such procedures on a suitable cell source, for example a bacterial, yeast, fungal or plant cell. Also possible is to use a probe of the invention to probe libraries made from yeast, bacterial, fungal or plant cells in order to obtain clones expressing variants or species homologues of the polypeptide of SEQ ID NO: 4, SEQ ID NO: 5, OR SEQ ID NO: 7. These clones can be manipulated by conventional techniques to generate a polypeptide of the invention which thereafter may be produced by recombinant or synthetic techniques known *per*

se.

The sequence of the polypeptide of SEQ ID NO: 4, SEQ ID NO: 5, OR SEQ ID NO: 7 and of variants and species homologues can also be modified to provide polypeptides of the invention. Amino acid substitutions may be made, for example from 1, 2 or 3 to 10, 20 or 30 substitutions. The same number of deletions and insertions may also be made. These changes may be made outside regions critical to the function of the polypeptide, as such a modified polypeptide will retain its proline specific endoprotease activity.

Polypeptides of the invention include fragments of the above mentioned full length polypeptides and of variants thereof, including fragments of the sequence set out in SEQ ID NO: 4, SEQ ID NO: 5, OR SEQ ID NO: 7. Such fragments will typically retain activity as an proline specific endoprotease. Fragments may be at least 50, 100 or 200 amino acids long or may be this number of amino acids short of the full length sequence shown in SEQ ID NO: 4, SEQ ID NO: 5, OR SEQ ID NO: 7.

Polypeptides of the invention can, if necessary, be produced by synthetic means although usually they will be made recombinantly as described below. Synthetic polypeptides may be modified, for example, by the addition of histidine residues or a T7 tag to assist their identification or purification, or by the addition of a signal sequence to promote their secretion from a cell.

Thus, the variants sequences may comprise those derived from strains of *Aspergillus* other than the strain from which the polypeptide of SEQ ID NO: 4, SEQ ID NO: 5, OR SEQ ID NO: 7 was isolated. Variants can be identified from other *Aspergillus* strains by looking for proline specific endoprotease activity and cloning and sequencing as described herein. Variants may include the deletion, modification or addition of single amino acids or groups of amino acids within the protein sequence, as long as the peptide maintains the basic biological functionality of the proline specific endoprotease of SEQ ID NO: 4, SEQ ID NO: 5, OR SEQ ID NO: 7.

Amino acid substitutions may be made, for example from 1, 2 or from 3 to 10, 20 or 30 substitutions. The modified polypeptide will generally retain activity as an proline specific endoprotease. Conservative substitutions may be made; such substitutions are well known in the art. Preferably substitutions do not affect the folding or activity of the polypeptide.

Shorter polypeptide sequences are within the scope of the invention. For example, a peptide of at least 50 amino acids or up to 60, 70, 80, 100, 150 or 200 amino acids in length is considered to fall within the scope of the invention as long as it demonstrates the basic biological functionality of the proline specific endoprotease of

SEQ ID NO: 4, SEQ ID NO: 5, OR SEQ ID NO: 7. In particular, but not exclusively, this aspect of the invention encompasses the situation in which the protein is a fragment of the complete protein sequence.

In a second embodiment, the present invention provides an to  
5 isolated polypeptide which has proline specific endoprotease activity, and is encoded  
by polynucleotides which hybridize or are capable of hybridizing under low stringency  
conditions, more preferably medium stringency conditions, and most preferably high  
stringency conditions, with (i) the nucleic acid sequence of SEQ ID NO: 1, SEQ ID NO:  
2, SEQ ID NO: 3 OR SEQ ID NO: 6 or a nucleic acid fragment comprising at least the  
10 c-terminal portion of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6,  
but having less than all or having bases differing from the bases of SEQ ID NO: 1, SEQ  
ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6; or (ii) with a nucleic acid strand  
complementary to SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6.

The term "capable of hybridizing" means that the target  
15 polynucleotide of the invention can hybridize to the nucleic acid used as a probe (for  
example, the nucleotide sequence set forth in SEQ. ID NO: 1, SEQ ID NO: 2, SEQ ID  
NO: 3 OR SEQ ID NO: 6, or a fragment thereof, or the complement of SEQ ID NO: 1,  
SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6) at a level significantly above  
background. The invention also includes the polynucleotides that encode the proline  
20 specific endoprotease of the invention, as well as nucleotide sequences which are  
complementary thereto. The nucleotide sequence may be RNA or DNA, including  
genomic DNA, synthetic DNA or cDNA. Preferably, the nucleotide sequence is DNA  
and most preferably, a genomic DNA sequence. Typically, a polynucleotide of the  
invention comprises a contiguous sequence of nucleotides which is capable of  
25 hybridizing under selective conditions to the coding sequence or the complement of the  
coding sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6.  
Such nucleotides can be synthesized according to methods well known in the art.

A polynucleotide of the invention can hybridize to the coding  
sequence or the complement of the coding sequence of SEQ ID NO: 1, SEQ ID NO: 2,  
30 SEQ ID NO: 3 OR SEQ ID NO: 6 at a level significantly above background.  
Background hybridization may occur, for example, because of other cDNAs present in  
a cDNA library. The signal level generated by the interaction between a polynucleotide  
of the invention and the coding sequence or complement of the coding sequence of  
SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6 is typically at least 10  
35 fold, preferably at least 20 fold, more preferably at least 50 fold, and even more  
preferably at least 100 fold, as intense as interactions between other polynucleotides

and the coding sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6. The intensity of interaction may be measured, for example, by radiolabelling the probe, for example with  $^{32}\text{P}$ . Selective hybridization may typically be achieved using conditions of low stringency (0.3M sodium chloride and 0.03M sodium citrate at about 40°C), medium stringency (for example, 0.3M sodium chloride and 0.03M sodium citrate at about 50°C) or high stringency (for example, 0.3M sodium chloride and 0.03M sodium citrate at about 60°C).

#### Modifications

Polynucleotides of the invention may comprise DNA or RNA. They may be single or double stranded. They may also be polynucleotides which include within them synthetic or modified nucleotides including peptide nucleic acids. A number of different types of modifications to polynucleotides are known in the art. These include a methylphosphonate and phosphorothioate backbones, and addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the polynucleotides described herein may be modified by any method available in the art.

It is to be understood that skilled persons may, using routine techniques, make nucleotide substitutions that do not affect the polypeptide sequence encoded by the polynucleotides of the invention to reflect the codon usage of any particular host organism in which the polypeptides of the invention are to be expressed.

The coding sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6 may be modified by nucleotide substitutions, for example from 1, 2 or 3 to 10, 25, 50 or 100 substitutions. The polynucleotide of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6 may alternatively or additionally be modified by one or more insertions and/or deletions and/or by an extension at either or both ends. The modified polynucleotide generally encodes a polypeptide which has proline specific endoprotease activity. Degenerate substitutions may be made and/or substitutions may be made which would result in a conservative amino acid substitution when the modified sequence is translated, for example as discussed with reference to polypeptides later.

#### Homologues

A nucleotide sequence which is capable of selectively hybridizing to the complement of the DNA coding sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6 is included in the invention and will generally have at least



50% or 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98% or at least 99% sequence identity to the coding sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6 over a region of at least 60, preferably at least 100, more preferably at least 200 contiguous nucleotides or most preferably over the full length of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6. Likewise, a nucleotide which encodes an active proline specific endoprotease and which is capable of selectively hybridizing to a fragment of a complement of the DNA coding sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6, is also embraced by the invention. A C-terminal fragment of the nucleic acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6 which is at least 80% or 90% identical over 60, preferably over 100 nucleotides, more preferably at least 90% identical over 200 nucleotides is encompassed by the invention.

Any combination of the above mentioned degrees of identity and minimum sizes may be used to define polynucleotides of the invention, with the more stringent combinations (i.e. higher identity over longer lengths) being preferred. Thus, for example, a polynucleotide which is at least 80% or 90% identical over 60, preferably over 100 nucleotides, forms one aspect of the invention, as does a polynucleotide which is at least 90% identical over 200 nucleotides.

The UWGCG Package provides the BESTFIT program which may be used to calculate identity (for example used on its default settings).

The PILEUP and BLAST N algorithms can also be used to calculate sequence identity or to line up sequences (such as identifying equivalent or corresponding sequences, for example on their default settings).

Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). This algorithm involves first identifying high scoring sequence pair (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold. These initial neighborhood word hits act as seeds for initiating searches to find HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extensions for the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T and X

determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a word length (W) of 11, the BLOSUM62 scoring matrix alignments (B) of 50, expectation (E) of 10, M=5, N=4, and a comparison of both strands.

The BLAST algorithm performs a statistical analysis of the similarity between two sequences. One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a sequence is considered similar to another sequence if the smallest sum probability in comparison of the first sequence to the second sequence is less than about 1, preferably less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

#### Primers and Probes

Polynucleotides of the invention include and may be used as primers, for example as polymerase chain reaction (PCR) primers, as primers for alternative amplification reactions, or as probes for example labelled with a revealing label by conventional means using radioactive or non-radioactive labels, or the polynucleotides may be cloned into vectors. Such primers, probes and other fragments will be at least 15, for example at least 20, 25, 30 or 40 nucleotides in length. They will typically be up to 40, 50, 60, 70, 100, 150, 200 or 300 nucleotides in length, or even up to a few nucleotides (such as 5 or 10 nucleotides) short of the coding sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6.

In general, primers will be produced by synthetic means, involving a step-wise manufacture of the desired nucleic acid sequence one nucleotide at a time. Techniques for accomplishing this using automated protocols are readily available in the art. Longer polynucleotides will generally be produced using recombinant means, for example using PCR cloning techniques. This will involve making a pair of primers (typically of about 15-30 nucleotides) to amplify the desired region of the proline specific endoprotease to be cloned, bringing the primers into contact with mRNA, cDNA or genomic DNA obtained from a yeast, bacterial, plant, prokaryotic or fungal cell, preferably of an *Aspergillus* strain, performing a polymerase chain reaction under conditions suitable for the amplification of the desired region, isolating the amplified fragment (e.g. by purifying the reaction mixture on an agarose gel) and recovering the amplified DNA. The primers may be designed to contain suitable restriction enzyme recognition sites so that the amplified DNA can be cloned into a suitable cloning vector.

Such techniques may be used to obtain all or part of the

polynucleotides encoding the proline specific endoprotease sequences described herein. Introns, promoter and trailer regions are within the scope of the invention and may also be obtained in an analogous manner (e.g. by recombinant means, PCR or cloning techniques), starting with genomic DNA from a fungal, yeast, bacterial plant or prokaryotic cell.

The polynucleotides or primers may carry a revealing label. Suitable labels include radioisotopes such as  $^{32}\text{P}$  or  $^{35}\text{S}$ , enzyme labels, or other protein labels such as biotin. Such labels may be added to polynucleotides or primers of the invention and may be detected using techniques known to persons skilled in the art.

Polynucleotides or primers (or fragments thereof) labelled or unlabelled may be used in nucleic acid-based tests for detecting or sequencing an proline specific endoprotease or a variant thereof in a fungal sample. Such detection tests will generally comprise bringing a fungal sample suspected of containing the DNA of interest into contact with a probe comprising a polynucleotide or primer of the invention under hybridizing conditions, and detecting any duplex formed between the probe and nucleic acid in the sample. Detection may be achieved using techniques such as PCR or by immobilizing the probe on a solid support, removing any nucleic acid in the sample which is not hybridized to the probe, and then detecting any nucleic acid which is hybridized to the probe. Alternatively, the sample nucleic acid may be immobilized on a solid support, the probe hybridized and the amount of probe bound to such a support after the removal of any unbound probe detected.

The probes of the invention may conveniently be packaged in the form of a test kit in a suitable container. In such kits the probe may be bound to a solid support where the assay format for which the kit is designed requires such binding. The kit may also contain suitable reagents for treating the sample to be probed, hybridizing the probe to nucleic acid in the sample, control reagents, instructions, and the like. The probes and polynucleotides of the invention may also be used in microassay.

Preferably, the polynucleotide of the invention is obtainable from the same organism as the polypeptide, such as a fungus, in particular a fungus of the genus *Aspergillus*.

The polynucleotides of the invention also include variants of the sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6 which encode for a polypeptide having proline specific endoprotease activity. Variants may be formed by additions, substitutions and/or deletions. Such variants of the coding sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6 may thus

encode polypeptides which have the ability to digest a polypeptide chain at the carboxyterminal side of proline.

#### Production of polynucleotides

5 Polynucleotides which do not have 100% identity with SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6 but fall within the scope of the invention can be obtained in a number of ways. Thus, variants of the proline specific endoprotease sequence described herein may be obtained for example, by probing genomic DNA libraries made from a range of organisms, such as those discussed as  
10 sources of the polypeptides of the invention. In addition, other fungal, plant or prokaryotic homologues of proline specific endoprotease may be obtained and such homologues and fragments thereof in general will be capable of hybridising to SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6. Such sequences may be obtained by probing cDNA libraries or genomic DNA libraries from other species, and  
15 probing such libraries with probes comprising all or part of SEQ ID. 1 under conditions of low, medium to high stringency (as described earlier). Nucleic acid probes comprising all or part of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6 may be used to probe cDNA or genomic libraries from other species, such as those described as sources for the polypeptides of the invention.

20 Species homologues may also be obtained using degenerate PCR, which uses primers designed to target sequences within the variants and homologues which encode conserved amino acid sequences. The primers can contain one or more degenerate positions and will be used at stringency conditions lower than those used for cloning sequences with single sequence primers against known sequences.

25 Alternatively, such polynucleotides may be obtained by site directed mutagenesis of the proline specific endoprotease sequences or variants thereof. This may be useful where, for example, silent codon changes to sequences are required to optimize codon preferences for a particular host cell in which the polynucleotide sequences are being expressed. Other sequence changes may be made in order to  
30 introduce restriction enzyme recognition sites, or to alter the property or function of the polypeptides encoded by the polynucleotides.

The invention includes double stranded polynucleotides comprising a polynucleotide of the invention and its complement.

35 The present invention also provides polynucleotides encoding the polypeptides of the invention described above. Since such polynucleotides will be useful as sequences for recombinant production of polypeptides of the invention, it is

not necessary for them to be capable of hybridising to the sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6, although this will generally be desirable. Otherwise, such polynucleotides may be labelled, used, and made as described above if desired.

5

#### Recombinant Polynucleotides.

The invention also provides vectors comprising a polynucleotide of the invention, including cloning and expression vectors, and in another aspect methods of growing, transforming or transfecting such vectors into a suitable host cell, for example under conditions in which expression of a polypeptide of, or encoded by a sequence of, the invention occurs. Provided also are host cells comprising a polynucleotide or vector of the invention wherein the polynucleotide is heterologous to the genome of the host cell. The term "heterologous", usually with respect to the host cell, means that the polynucleotide does not naturally occur in the genome of the host cell or that the polypeptide is not naturally produced by that cell. Preferably, the host cell is a yeast cell, for example a yeast cell of the genus *Kluyveromyces* or *Saccharomyces* or a filamentous fungal cell, for example of the genus *Aspergillus*.

Polynucleotides of the invention can be incorporated into a recombinant replicable vector, for example a cloning or expression vector. The vector may be used to replicate the nucleic acid in a compatible host cell. Thus, in a further embodiment, the invention provides a method of making polynucleotides of the invention by introducing a polynucleotide of the invention into a replicable vector, introducing the vector into a compatible host cell, and growing the host cell under conditions which bring about replication of the vector. The vector may be recovered from the host cell. Suitable host cells are described below in connection with expression vectors.

#### Vectors

The vector into which the expression cassette of the invention is inserted may be any vector that may conveniently be subjected to recombinant DNA procedures, and the choice of the vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extra-chromosomal entity, the replication of which is independent of chromosomal replication, such as a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicates together with the chromosome(s) into which it has been

integrated.

Preferably, when a polynucleotide of the invention is in a vector it is operably linked to a regulatory sequence which is capable of providing for the expression of the coding sequence by the host cell, i.e. the vector is an expression vector. The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence such as a promoter, enhancer or other expression regulation signal "operably linked" to a coding sequence is positioned in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

The vectors may, for example in the case of plasmid, cosmid, virus or phage vectors, be provided with an origin of replication, optionally a promoter for the expression of the polynucleotide and optionally an enhancer and/or a regulator of the promoter. A terminator sequence may be present, as may be a polyadenylation sequence. The vectors may contain one or more selectable marker genes, for example an ampicillin resistance gene in the case of a bacterial plasmid or a neomycin resistance gene for a mammalian vector. Vectors may be used *in vitro*, for example for the production of RNA or can be used to transfect or transform a host cell.

The DNA sequence encoding the polypeptide is preferably introduced into a suitable host as part of an expression construct in which the DNA sequence is operably linked to expression signals which are capable of directing expression of the DNA sequence in the host cells. For transformation of the suitable host with the expression construct transformation procedures are available which are well known to the skilled person. The expression construct can be used for transformation of the host as part of a vector carrying a selectable marker, or the expression construct is co-transformed as a separate molecule together with the vector carrying a selectable marker. The vectors may contain one or more selectable marker genes.

Preferred selectable markers include but are not limited to those that complement a defect in the host cell or confer resistance to a drug. They include for example versatile marker genes that can be used for transformation of most filamentous fungi and yeasts such as acetamidase genes or cDNAs (the *amdS*, *niaD*, *facA* genes or cDNAs from *A.nidulans*, *A.oryzae*, or *A.niger*), or genes providing resistance to antibiotics like G418, hygromycin, bleomycin, kanamycin, phleomycin or benomyl resistance (*benA*). Alternatively, specific selection markers can be used such as auxotrophic markers which require corresponding mutant host strains: e.g. *URA3*

(from *S.cerevisiae* or analogous genes from other yeasts), pyrG or pyrA (from *A.nidulans* or *A.niger*), argB (from *A.nidulans* or *A.niger*) or trpC. In a preferred embodiment the selection marker is deleted from the transformed host cell after introduction of the expression construct so as to obtain transformed host cells capable of producing the polypeptide which are free of selection marker genes.

Other markers include ATP synthetase subunit 9 (oliC), orotidine-5'-phosphate- decarboxylase (pvrA), the bacterial G418 resistance gene (useful in yeast, but not in filamentous fungi), the ampicillin resistance gene (*E. coli*), the neomycin resistance gene (*Bacillus*) and the *E. coli* uidA gene, coding for glucuronidase (GUS). Vectors may be used *in vitro*, for example for the production of RNA or to transfect or transform a host cell.

For most filamentous fungi and yeast, the expression construct is preferably integrated into the genome of the host cell in order to obtain stable transformants. However, for certain yeasts suitable episomal vector systems are also available into which the expression construct can be incorporated for stable and high level expression. Examples thereof include vectors derived from the 2  $\mu$ m, CEN and pKD1 plasmids of *Saccharomyces* and *Kluyveromyces*, respectively, or vectors containing an AMA sequence (e.g. AMA1 from *Aspergillus*). When expression constructs are integrated into host cell genomes, the constructs are either integrated at random loci in the genome, or at predetermined target loci using homologous recombination, in which case the target loci preferably comprise a highly expressed gene. A highly expressed gene is a gene whose mRNA can make up at least 0.01% (w/w) of the total cellular mRNA, for example under induced conditions, or alternatively, a gene whose gene product can make up at least 0.2% (w/w) of the total cellular protein, or, in case of a secreted gene product, can be secreted to a level of at least 0.05 g/l.

An expression construct for a given host cell will usually contain the following elements operably linked to each other in consecutive order from the 5'-end to 3'-end relative to the coding strand of the sequence encoding the polypeptide of the first aspect: (1) a promoter sequence capable of directing transcription of the DNA sequence encoding the polypeptide in the given host cell, (2) preferably, a 5'-untranslated region (leader), (3) optionally, a signal sequence capable of directing secretion of the polypeptide from the given host cell into the culture medium, (4) the DNA sequence encoding a mature and preferably active form of the polypeptide, and preferably also (5) a transcription termination region (terminator) capable of terminating transcription downstream of the DNA sequence encoding the polypeptide.

Downstream of the DNA sequence encoding the polypeptide, the expression construct preferably contains a 3' untranslated region containing one or more transcription termination sites, also referred to as a terminator. The origin of the terminator is less critical. The terminator can for example be native to the DNA sequence encoding the polypeptide. However, preferably a yeast terminator is used in yeast host cells and a filamentous fungal terminator is used in filamentous fungal host cells. More preferably, the terminator is endogenous to the host cell in which the DNA sequence encoding the polypeptide is expressed.

Enhanced expression of the polynucleotide encoding the polypeptide of the invention may also be achieved by the selection of heterologous regulatory regions, e.g. promoter, signal sequence and terminator regions, which serve to increase expression and, if desired, secretion levels of the protein of interest from the chosen expression host and/or to provide for the inducible control of the expression of the polypeptide of the invention.

Aside from the promoter native to the gene encoding the polypeptide of the invention, other promoters may be used to direct expression of the polypeptide of the invention. The promoter may be selected for its efficiency in directing the expression of the polypeptide of the invention in the desired expression host.

Promoters/enhancers and other expression regulation signals may be selected to be compatible with the host cell for which the expression vector is designed. For example prokaryotic promoters may be used, in particular those suitable for use in *E.coli* strains. When expression of the polypeptides of the invention is carried out in mammalian cells, mammalian promoters may be used. Tissues-specific promoters, for example hepatocyte cell-specific promoters, may also be used. Viral promoters may also be used, for example the Moloney murine leukaemia virus long terminal repeat (MMLV LTR), the rous sarcoma virus (RSV) LTR promoter, the SV40 promoter, the human cytomegalovirus (CMV) IE promoter, herpes simplex virus promoters or adenovirus promoters.

Suitable yeast promoters include the *S. cerevisiae* GAL4 and ADH promoters and the *S. pombe* nmt1 and adh promoter. Mammalian promoters include the metallothionein promoter which can be induced in response to heavy metals such as cadmium. Viral promoters such as the SV40 large T antigen promoter or adenovirus promoters may also be used. All these promoters are readily available in the art.

Mammalian promoters, such as  $\beta$ -actin promoters, may be used. Tissue-specific promoters, in particular endothelial or neuronal cell specific promoters (for example the DDAH I and DDAH II promoters), are especially preferred. Viral



promoters may also be used, for example the Moloney murine leukaemia virus long terminal repeat (MMLV LTR), the rous sarcoma virus (RSV) LTR promoter, the SV40 promoter, the human cytomegalovirus (CMV) IE promoter, adenovirus, HSV promoters (such as the HSV IE promoters), or HPV promoters, particularly the HPV upstream regulatory region (URR). Viral promoters are readily available in the art.

A variety of promoters can be used that are capable of directing transcription in the host cells of the invention. Preferably the promoter sequence is derived from a highly expressed gene as previously defined. Examples of preferred highly expressed genes from which promoters are preferably derived and/or which are comprised in preferred predetermined target loci for integration of expression constructs, include but are not limited to genes encoding glycolytic enzymes such as triose-phosphate isomerases (TPI), glyceraldehyde-phosphate dehydrogenases (GAPDH), phosphoglycerate kinases (PGK), pyruvate kinases (PYK), alcohol dehydrogenases (ADH), as well as genes encoding amylases, glucoamylases, proteases, xylanases, cellobiohydrolases,  $\beta$ -galactosidases, alcohol (methanol) oxidases, elongation factors and ribosomal proteins. Specific examples of suitable highly expressed genes include e.g. the LAC4 gene from *Kluyveromyces* sp., the methanol oxidase genes (AOX and MOX) from *Hansenula* and *Pichia*, respectively, the glucoamylase (glaA) genes from *A.niger* and *A.awamori*, the *A.oryzae* TAKA-amylase gene, the *A.nidulans* gpdA gene and the *T.reesei* cellobiohydrolase genes.

Examples of strong constitutive and/or inducible promoters which are preferred for use in fungal expression hosts are those which are obtainable from the fungal genes for xylanase (xlnA), phytase, ATP-synthetase subunit 9 (oliC), triose phosphate isomerase (tpi), alcohol dehydrogenase (AdhA), amylase (amy), amyloglucosidase (AG - from the glaA gene), acetamidase (amdS) and glyceraldehyde-3-phosphate dehydrogenase (gpd) promoters.

Examples of strong yeast promoters which may be used include those obtainable from the genes for alcohol dehydrogenase, lactase, 3-phosphoglycerate kinase and triosephosphate isomerase.

Examples of strong bacterial promoters which may be used include the amylase and SPo2 promoters as well as promoters from extracellular protease genes.

Promoters suitable for plant cells which may be used include napaline synthase (nos), octopine synthase (ocs), mannopine synthase (mas), ribulose small subunit (rubisco ssu), histone, rice actin, phaseolin, cauliflower mosaic virus (CMV) 35S and 19S and circovirus promoters. The vector may further include

sequences flanking the polynucleotide giving rise to RNA which comprise sequences homologous to ones from eukaryotic genomic sequences, preferably mammalian genomic sequences, or viral genomic sequences. This will allow the introduction of the polynucleotides of the invention into the genome of eukaryotic cells or viruses by homologous recombination. In particular, a plasmid vector comprising the expression cassette flanked by viral sequences can be used to prepare a viral vector suitable for delivering the polynucleotides of the invention to a mammalian cell. Other examples of suitable viral vectors include herpes simplex viral vectors and retroviruses, including lentiviruses, adenoviruses, adeno-associated viruses and HPV viruses (such as HPV-16 or HPV-18). Gene transfer techniques using these viruses are known to those skilled in the art. Retrovirus vectors for example may be used to stably integrate the polynucleotide giving rise to the antisense RNA into the host genome. Replication-defective adenovirus vectors by contrast remain episomal and therefore allow transient expression.

The vector may contain a polynucleotide of the invention oriented in an antisense direction to provide for the production of antisense RNA. This may be used to reduce, if desirable, the levels of expression of the polypeptide.

#### Host Cells and Expression

In a further aspect the invention provides a process for preparing a polypeptide of the invention which comprises cultivating a host cell transformed or transfected with an expression vector as described above under conditions suitable for expression by the vector of a coding sequence encoding the polypeptide, and recovering the expressed polypeptide. Polynucleotides of the invention can be incorporated into a recombinant replicable vector, such as an expression vector. The vector may be used to replicate the nucleic acid in a compatible host cell. Thus in a further embodiment, the invention provides a method of making a polynucleotide of the invention by introducing a polynucleotide of the invention into a replicable vector, introducing the vector into a compatible host cell, and growing the host cell under conditions which bring about the replication of the vector. The vector may be recovered from the host cell. Suitable host cells include bacteria such as *E. coli*, yeast, mammalian cell lines and other eukaryotic cell lines, for example insect cells such as Sf9 cells and (e.g. filamentous) fungal cells.

Preferably the polypeptide is produced as a secreted protein in which case the DNA sequence encoding a mature form of the polypeptide in the expression construct is operably linked to a DNA sequence encoding a signal sequence. In the

case where the gene encoding the secreted protein has in the wild type strain a signal sequence preferably the signal sequence used will be native (homologous) to the DNA sequence encoding the polypeptide. Alternatively the signal sequence is foreign (heterologous) to the DNA sequence encoding the polypeptide, in which case the signal sequence is preferably endogenous to the host cell in which the DNA sequence is expressed. Examples of suitable signal sequences for yeast host cells are the signal sequences derived from yeast MFalpha genes. Similarly, a suitable signal sequence for filamentous fungal host cells is e.g. a signal sequence derived from a filamentous fungal amyloglucosidase (AG) gene, e.g. the *A.niger* glaA gene. This signal sequence may be used in combination with the amyloglucosidase (also called (gluco)amylase) promoter itself, as well as in combination with other promoters. Hybrid signal sequences may also be used within the context of the present invention.

Preferred heterologous secretion leader sequences are those originating from the fungal amyloglucosidase (AG) gene (glaA - both 18 and 24 amino acid versions e.g. from *Aspergillus*), the MFalpha gene (yeasts e.g. *Saccharomyces* and *Kluyveromyces*) or the alpha-amylase gene (*Bacillus*).

The vectors may be transformed or transfected into a suitable host cell as described above to provide for expression of a polypeptide of the invention. This process may comprise culturing a host cell transformed with an expression vector as described above under conditions suitable for expression of the polypeptide, and optionally recovering the expressed polypeptide.

A further aspect of the invention thus provides host cells transformed or transfected with or comprising a polynucleotide or vector of the invention. Preferably the polynucleotide is carried in a vector which allows the replication and expression of the polynucleotide. The cells will be chosen to be compatible with the said vector and may for example be prokaryotic (for example bacterial), or eukaryotic fungal, yeast or plant cells.

The invention encompasses processes for the production of a polypeptide of the invention by means of recombinant expression of a DNA sequence encoding the polypeptide. For this purpose the DNA sequence of the invention can be used for gene amplification and/or exchange of expression signals, such as promoters, secretion signal sequences, in order to allow economic production of the polypeptide in a suitable homologous or heterologous host cell. A homologous host cell is herein defined as a host cell which is of the same species or which is a variant within the same species as the species from which the DNA sequence is derived.

Suitable host cells are preferably prokaryotic microorganisms such as

bacteria, or more preferably eukaryotic organisms, for example fungi, such as yeasts or filamentous fungi, or plant cells. In general, yeast cells are preferred over filamentous fungal cells because they are easier to manipulate. However, some proteins are either poorly secreted from yeasts, or in some cases are not processed properly (e.g. hyperglycosylation in yeast). In these instances, a filamentous fungal host organism should be selected.

Bacteria from the genus *Bacillus* are very suitable as heterologous hosts because of their capability to secrete proteins into the culture medium. Other bacteria suitable as hosts are those from the genera *Streptomyces* and *Pseudomonas*.

A preferred yeast host cell for the expression of the DNA sequence encoding the polypeptide is one of the genus *Saccharomyces*, *Kluyveromyces*, *Hansenula*, *Pichia*, *Yarrowia*, or *Schizosaccharomyces*. More preferably, a yeast host cell is selected from the group consisting of the species *Saccharomyces cerevisiae*, *Kluyveromyces lactis* (also known as *Kluyveromyces marxianus* var. *lactis*), *Hansenula polymorpha*, *Pichia pastoris*, *Yarrowia lipolytica*, and *Schizosaccharomyces pombe*.

Most preferred for the expression of the DNA sequence encoding the polypeptide are, however, filamentous fungal host cells. Preferred filamentous fungal host cells are selected from the group consisting of the genera *Aspergillus*, *Trichoderma*, *Fusarium*, *Disporotrichum*, *Penicillium*, *Acremonium*, *Neurospora*, *Thermoascus*, *Myceliophthora*, *Sporotrichum*, *Thielavia*, and *Talaromyces*. More preferably a filamentous fungal host cell is of the species *Aspergillus oysae*, *Aspergillus sojae* or *Aspergillus nidulans* or is of a species from the *Aspergillus niger* Group (as defined by Raper and Fennell, The Genus *Aspergillus*, The Williams & Wilkins Company, Baltimore, pp 293-344, 1965). These include but are not limited to *Aspergillus niger*, *Aspergillus awamori*, *Aspergillus tubigensis*, *Aspergillus aculeatus*, *Aspergillus foetidus*, *Aspergillus nidulans*, *Aspergillus japonicus*, *Aspergillus oryzae* and *Aspergillus ficuum*, and also those of the species *Trichoderma reesei*, *Fusarium graminearum*, *Penicillium chrysogenum*, *Acremonium alabamense*, *Neurospora crassa*, *Myceliophthora thermophilum*, *Sporotrichum cellulophilum*, *Disporotrichum dimorphosporum* and *Thielavia terrestris*.

Examples of preferred expression hosts within the scope of the present invention are fungi such as *Aspergillus* species (in particular those described in EP-A-184,438 and EP-A-284,603) and *Trichoderma* species; bacteria such as *Bacillus* species (in particular those described in EP-A-134,048 and EP-A-253,455), especially *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Pseudomonas* species; and yeasts such as *Kluyveromyces* species (in particular those described in

EP-A-096,430 such as *Kluyveromyces lactis* and in EP-A-301,670) and *Saccharomyces* species, such as *Saccharomyces cerevisiae*.

Host cells according to the invention include plant cells, and the invention therefore extends to transgenic organisms, such as plants and parts thereof, which contain one or more cells of the invention. The cells may heterologously express the polypeptide of the invention or may heterologously contain one or more of the polynucleotides of the invention. The transgenic (or genetically modified) plant may therefore have inserted (typically stably) into its genome a sequence encoding the polypeptides of the invention. The transformation of plant cells can be performed using known techniques, for example using a Ti or a Ri plasmid from *Agrobacterium tumefaciens*. The plasmid (or vector) may thus contain sequences necessary to infect a plant, and derivatives of the Ti and/or Ri plasmids may be employed.

The host cell may overexpress the polypeptide, and techniques for engineering over-expression are well known and can be used in the present invention. The host may thus have two or more copies of the polynucleotide.

Alternatively, direct infection of a part of a plant, such as a leaf, root or stem can be effected. In this technique the plant to be infected can be wounded, for example by cutting the plant with a razor, puncturing the plant with a needle or rubbing the plant with an abrasive. The wound is then inoculated with the *Agrobacterium*. The plant or plant part can then be grown on a suitable culture medium and allowed to develop into a mature plant. Regeneration of transformed cells into genetically modified plants can be achieved by using known techniques, for example by selecting transformed shoots using an antibiotic and by sub-culturing the shoots on a medium containing the appropriate nutrients, plant hormones and the like.

#### Culture of host cells and recombinant production

The invention also includes cells that have been modified to express the proline specific endoprotease or a variant thereof. Such cells include transient, or preferably stably modified higher eukaryotic cell lines, such as mammalian cells or insect cells, lower eukaryotic cells, such as yeast and filamentous fungal cells or prokaryotic cells such as bacterial cells.

It is also possible for the polypeptides of the invention to be transiently expressed in a cell line or on a membrane, such as for example in a baculovirus expression system. Such systems, which are adapted to express the proteins according to the invention, are also included within the scope of the present invention.

According to the present invention, the production of the polypeptide of the invention can be effected by the culturing of microbial expression hosts, which have been transformed with one or more polynucleotides of the present invention, in a conventional nutrient fermentation medium.

5           The recombinant host cells according to the invention may be cultured using procedures known in the art. For each combination of a promoter and a host cell, culture conditions are available which are conducive to the expression the DNA sequence encoding the polypeptide. After reaching the desired cell density or titre of the polypeptide the culturing is ceased and the polypeptide is recovered using  
10   known procedures.

          The fermentation medium can comprise a known culture medium containing a carbon source (e.g. glucose, maltose, molasses, etc.), a nitrogen source (e.g. ammonium sulphate, ammonium nitrate, ammonium chloride, etc.), an organic nitrogen source (e.g. yeast extract, malt extract, peptone, etc.) and inorganic nutrient  
15   sources (e.g. phosphate, magnesium, potassium, zinc, iron, etc.). Optionally, an inducer (dependent on the expression construct used) may be included or subsequently be added.

          The selection of the appropriate medium may be based on the choice of expression host and/or based on the regulatory requirements of the expression  
20   construct. Suitable media are well-known to those skilled in the art. The medium may, if desired, contain additional components favoring the transformed expression hosts over other potentially contaminating microorganisms.

          The fermentation may be performed over a period of from 0.5-30 days. Fermentation may be a batch, continuous or fed-batch process, at a suitable  
25   temperature in the range of between 0°C and 45°C and, for example, at a pH from 2 to 10. Preferred fermentation conditions include a temperature in the range of between 20°C and 37°C and/or a pH between 3 and 9. The appropriate conditions are usually selected based on the choice of the expression host and the protein to be expressed.

          After fermentation, if necessary, the cells can be removed from the  
30   fermentation broth by means of centrifugation or filtration. After fermentation has stopped or after removal of the cells, the polypeptide of the invention may then be recovered and, if desired, purified and isolated by conventional means. The proline specific endoprotease of the invention can be purified from fungal mycelium or from the culture broth into which the proline specific endoprotease is released by the cultured  
35   fungal cells.

In a preferred embodiment the polypeptide is obtained from a fungus, more preferably from an *Aspergillus*, most preferably from *Aspergillus niger*.

#### Modifications

5 Polypeptides of the invention may be chemically modified, e.g. post-translationally modified. For example, they may be glycosylated (one or more times) or comprise modified amino acid residues. They may also be modified by the addition of histidine residues to assist their purification or by the addition of a signal sequence to promote secretion from the cell. The polypeptide may have amino- or carboxyl-  
10 terminal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about 20-25 residues, or a small extension that facilitates purification, such as a poly-histidine tract, an antigenic epitope or a binding domain.

A polypeptide of the invention may be labelled with a revealing label. The revealing label may be any suitable label which allows the polypeptide to be  
15 detected. Suitable labels include radioisotopes, e.g.  $^{125}\text{I}$ ,  $^{35}\text{S}$ , enzymes, antibodies, polynucleotides and linkers such as biotin.

The polypeptides may be modified to include non-naturally occurring amino acids or to increase the stability of the polypeptide. When the proteins or peptides are produced by synthetic means, such amino acids may be introduced during  
20 production. The proteins or peptides may also be modified following either synthetic or recombinant production.

The polypeptides of the invention may also be produced using D-amino acids. In such cases the amino acids will be linked in reverse sequence in the C to N orientation. This is conventional in the art for producing such proteins or peptides.

25 A number of side chain modifications are known in the art and may be made to the side chains of the proteins or peptides of the present invention. Such modifications include, for example, modifications of amino acids by reductive alkylation by reaction with an aldehyde followed by reduction with  $\text{NaBH}_4$ , amidination with methylacetimidate or acylation with acetic anhydride.

30 The sequences provided by the present invention may also be used as starting materials for the construction of "second generation" enzymes. "Second generation" proline specific proteases are proline specific proteases, altered by mutagenesis techniques (e.g. site-directed mutagenesis), which have properties that differ from those of wild-type proline specific protease or recombinant proline specific  
35 proteases such as those produced by the present invention. For example, their temperature or pH optimum, specific activity, substrate affinity or thermostability may

be altered so as to be better suited for use in a particular process.

Amino acids essential to the activity of the proline specific endoprotease of the invention, and therefore preferably subject to substitution, may be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis. In the latter technique mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological activity (e.g. proline specific endoprotease activity) to identify amino acid residues that are critical to the activity of the molecule. Sites of enzyme-substrate interaction can also be determined by analysis of crystal structure as determined by such techniques as nuclear magnetic resonance, crystallography or photo-affinity labelling.

The use of yeast and filamentous fungal host cells is expected to provide for such post-translational modifications (e.g. proteolytic processing, myristilation, glycosylation, truncation, and tyrosine, serine or threonine phosphorylation) as may be needed to confer optimal biological activity on recombinant expression products of the invention.

#### Preparations

Polypeptides of the invention may be in an isolated form. It will be understood that the polypeptide may be mixed with carriers or diluents which will not interfere with the intended purpose of the polypeptide and still be regarded as isolated. A polypeptide of the invention may also be in a substantially purified form, in which case it will generally comprise the polypeptide in a preparation in which more than 70%, e.g. more than 80%, 90%, 95%, 98% or 99% of the proteins in the preparation is a polypeptide of the invention.

Polypeptides of the invention may be provided in a form such that they are outside their natural cellular environment. Thus, they may be substantially isolated or purified, as discussed above, or in a cell in which they do not occur in nature, for example a cell of other fungal species, animals, plants or bacteria. Furthermore the polypeptides according to the invention may be used in an immobilized form so that large quantities of protein containing liquids can be treated. Ways to select appropriate support materials and suitable immobilization methods have been extensively described in the literature, for example in "Immobilization of Enzymes and Cells" (ed. Gordon F. Bickerstaff; ISBN 0-89603-386-4).

The invention also relates to the use of a prolyl-specific endoprotease in the preparation of a beverage. A prolyl-specific endoprotease is used preferably in



the preparation of beer, wine or fruit juice. By the addition of a prolyl-specific endoprotease according to the method according to the invention, a reduction or prevention of haze is achieved. By adding these prolyl-specific endoproteases, new beverage are obtained. Thus, the invention also relates to beverages obtainable by the method according to the invention. These beverages include for example beer, wine and fruit juice obtainable by a method according to the invention.

An advantage of the beverages obtainable by the method according to the invention is that these beverages have a high content of anti-oxidants.

Polyphenols are anti-oxidants. Beer is usually treated with a polyphenol-removing agent to prevent the formation of haze, and as a result the beer obtained has a low antioxidant activity. The same is true for other beverages treated with poly-phenol removing agents. Beer obtainable by the method according to the invention has a higher endogenous anti-oxidant activity. Because anti-oxidants are seen as health improving ingredients, the beverages obtainable by the method according to the invention may be considered as beverages that are healthier than the same type of beverage prepared with polyphenol removing agents, such as PVPP.

It is another advantage of the method according to the invention that it prevents the loss of hydrophobic polypeptides during fermentation and conditioning of high gravity beers. The improved extraction of hydrophobic polypeptides during the mashing phase of beer production thus results in higher yields and improved beer head stabilities. (Brey *et al*; Journal of the Institute of Brewing, Vol .108, No. 4, 424-433, 2002). Proline-specific endoproteases are known to reduce the hydrophobicity of peptides hereby improving their water solubility and diminishing their bitter off-tastes (WO 02/45523). In fact we demonstrate higher protein levels and improved beer head stabilities upon the use of Endo-Pro enzyme in Example 15. The use of proline-specific endoproteases optionally in combination with the auxillary proteases specified in the present application is therefore advantageous.

It is yet another advantage of the method according to the invention that the color of fruit juices obtainable by the method is not or less faded than the color of fruit juices obtained after the removal of polyphenols. Wines and fruit juices obtainable by the method according to the invention have improved aroma and flavour in comparison to beverages obtained by a method wherein bentonite or a similar compound is used, since bentonite not only removes proteins but also aroma and/or flavour components.

## EXAMPLES

### EXAMPLE 1

#### 5 Materials

##### Proline-specific endoprotease enzymes (Endo-Pro's)

Aspergillus niger G306 was deposited with the CBS (CBS109712) on 10 September 2001. A niger G306 contains a gene encoding a prolyl-specific endoprotease according to the invention. The gene or cDNA obtainable from this  
10 organism may be cloned and expressed in any Aspergillus niger host using known methods.

The following samples were used:

- 1) "Endo-Pro A", a proline-specific endoprotease was used in experiments with beer.  
15 The sample was an ultrafiltration concentrate obtained after ultrafiltration of a fermentation broth obtained after fermentation of an Aspergillus niger strain comprising a gene coding for a proline-specific endoprotease. The prolyl-specific activity of the Endo-Pro A sample was 5.06 U/ml, determined as described under Methods. The protein concentration was estimated to be 50g/l, based on the specific activity of a  
20 sample of prolyl-specific endoprotease with a purity higher than 90%.
- 2) "Endo-Pro B", an proline-specific endoprotease was used in experiments with wine. The sample was obtained after purification over a column and had an activity of 6.0 U/ml.

#### 25 Papain

Collupulin, a liquid papain preparation commercially available from DSM was used for experiments with papain. The activity is 5280 NF/mg. One unit NF is the quantity of papain activity that catalyzes the hydrolysis of casein to produce one microgramme equivalent of soluble tyrosine per hour at pH 6.0. The protein  
30 concentration in the papain sample was measured, which is 119 g/l (Lowry).

##### Polyvinylpyrrolidone (PVPP)

PVPP used was a commercially available non-water soluble PVPP under the name 'Polyclar AT'.

### Beer

A malt beer (pilsener type) from "Les Trois Brasseurs" in Lille, France, was used in all experiment performed with beer. The alcohol percentage of this beer was 5.2% (v/v) and the pH was 4.4. This particular beer was chosen because of the relative high amount of turbidity measured in this beer upon chilling, in comparison with other commercially available beers. The beer had a protein concentration of 0.9 g/l, as determined by Lowry's-method.

### White wine

A white wine prepared from white Sauvignon grapes was used without any protein removal treatment. The alcoholic fermentation during wine preparation was performed with a selected yeast VL3 from Lallemmand. The oenologic analysis of the wine gave the following results:

Sugars (g/l)	1.1
Ethanol %vol	12.97
Total Acidity (g H <sub>2</sub> SO <sub>4</sub> /l)	4.14
Volatil acidity (g H <sub>2</sub> SO <sub>4</sub> /l)	0.22
PH	3.46
Free SO <sub>2</sub> (mg/l)	18
Total SO <sub>2</sub> (mg/l)	96
Glycerol (g/l)	3
Tartric acid (g/l)	3
Malic acid (g/l)	2.8
Lactic acid (g/l)	0.1
Level of Folin	7

### **Example 2**

#### Methods

##### Spectrophotometric method for determining enzyme activities

To measure the activity of the proline-specific endoprotease, a substrate solution of 2 mM solution of N-carbobenzoxy-glycine-proline-p-nitro anilide (Z-Gly-Pro-pNA; m.w. 426.43 ; Bachem, Switzerland) made in a 0.1 M citric acid / 0.2

M disodium phosphate buffer pH 5.0 containing 40 % dioxan is used.

To 1 mL of buffer pH 5.0, 250 µl of the substrate solution is added followed by 100 µl of the enzyme solution (larger or smaller volume amounts of enzyme solution should be compensated for by buffer solution). The reaction mixture is incubated at 37°C and the release of pNA is followed by measuring the absorbance increase at 410 nm.

One unit is defined as the enzyme activity that liberates 1 µmol pNA from Z-Gly-Pro-pNA in 1 minute under described reaction conditions using a molar extinction coefficient (E) of 8,800 M<sup>-1</sup>.

The activity of the glycine-specific endoprotease isolated from Flavourzyme 1000 L (NOVO, Denmark) was measured on the synthetic chromogenic substrate Z-Gly-Gly-pNA at pH 5.0 and 37 degrees C using procedures as described. One unit of glycine-specific endoprotease is defined as the quantity of enzyme that provokes the release of 1 micromol of pNA from Z-Gly-Gly-pNA per minute at pH 5.0 and 37 degrees C.

The activity of the proline-specific carboxypeptidase from *Xanthomonas* species was measured by measuring the quantity of proline residues released from the synthetic peptide Z-Pro-Pro (Bachem, Switzerland) using an amino acid analyzer. One unit is the quantity of enzyme that provokes the release of 1 micromol of proline from Z-Pro-Pro per hour at pH 7.0 and 37 degrees C.

#### Chill Haze measurements (Alcohol/low-temperature test according to Lucien Chapon)

Turbidity or haze was measured with a turbidimeter ("Tannometer", Pfeuffer GmbH, Kitzingen, Germany) in line with the operating instructions. In cold beer a reversible turbidity can occur which is caused by precipitated polyphenol-protein complexes. The addition of extra alcohol accelerates the formation of these turbidities.

In the "chill haze test" this phenomenon is used to quantitate the polyphenol-protein complexes present. To calibrate the Tannometer, a formazine standard solution was prepared as described (Jean de Clerk, "Cours de Brasseries" 2nd Edition, Vol 2, 1963, pp.595-596, Université de Louvain, Belgium). The beer haze or turbidity unit used is the ECB which is nephelometric turbidity units as recommended by the European

Brewery Convention. Chill haze tests as described for beer may also be performed with alcohol free beers or worts (adapted from the Operating Instruction Guide of the Tannometer). In these cases ethanol is also added to the samples in an amount sufficient to reach an alcohol content of 10% (v/v) in the samples. Ethanol was added to all wort samples to reach 10% (v/v) after which each sample is cooled to -8°C during 30 min. Then the haze formed (Turbidity, in units EBC) is quickly measured in the turbidimeter with its measuring chamber also at -8°C.

"Hot haze" measurements according to the European Brewery Convention.

The "hot haze test" is a beer haze measuring protocol as recommended by the European Brewery Convention under number 9.30. It has been shown that storage of beer at elevated temperatures for relatively short periods of time will result in a haze level similar to that found in the same beer after prolonged storage at room temperature. This "hot haze" test is performed by cooling the beer overnight in the cooling bath at 0°C and reading the initial turbidity in the morning. Then the beer is placed in a bath at 60°C for 48h without agitation. Finally the beer is cooled and kept at 0°C overnight before a final turbidity measurement at 0°C.

Heating haze tests for wine and fruit juices.

As described by K.J. Siebert (K.J. Siebert et al, J. Agric. Food Chem. 44 (1996)) haze in beverages like wine or fruit juices can be induced by a heating test. The amount of haze formed is mainly a function of the levels of haze-active proteins and polyphenols in the beverage. In the Heating test, the turbidity of samples (of for example wine or fruit juice) is measured with a turbidimeter before and after heating at 80°C during 30 min. Before measuring the turbidity, the heated sample is cooled down under cold water until a temperature of 22-25 °C is reached. In the wine trials (see Example 7) the calibration of the turbidimeter was performed with NTU-formazine standard solutions, for the fruit juice trials (see Example 8) the NTU turbidity standard solution was purchased from Reagecon, Ireland. NTU= nephelometric turbidity units.

Control experiments

1. A blank experiment was performed wherein no exogenous protein was added during the incubation.
2. An experiment was carried out wherein beer was used that had been treated with a large amount of PVPP (1000 g/hl) before incubation. This experiment allowed the determination of the average amount of haze induced by the chill haze test which is due to polyphenol-protein precipitate, because PVPP removes polyphenols from the beer, and thus interferes with formation of haze.
3. Experiments were performed wherein exogenous proteins (prolyl-specific endoprotease or papain, respectively) were added to beer cooled to 0°C after incubation at 40°C for 1 hour. Incubation at 0°C took place for 15 minutes prior to haze measurements. Since the enzyme and papain are not or hardly active at 0°C, these experiments allowed discrimination between the enzyme activity

effect and non-enzymatic protein effects.

### LC/MS analysis

HPLC using a P4000 pump (Thermoquest™, Breda, the Netherlands) equipped with an LCQ ion trap mass spectrometer (Thermoquest™, Breda, the Netherlands) was used ° for the characterization of the three synthetic peptides, which were separated using a 150 \* 1mm PEPMAP C18 300A (LC Packings, Amsterdam, The Netherlands) column in combination with a gradient of 0.1% formic acid in Milli Q water (Millipore, Bedford, MA, USA; Solution A) and 0.1% formic acid in acetonitrile (Solution B) for elution.

Detailed information on the individual peptides was obtained by using the "scan dependent" MS/MS algorithm which is a characteristic algorithm for an ion trap mass spectrometer. The endoprotease specificity for hydroxy proline and alanine was checked by comparison of the experimental peptide sequences of the different peptides, obtained with MS/MS, with the theoretical sequence information.

### **Example 3**

#### Effects of the addition of a prolyl-specific endoprotease on haze formation in beer

To a decarbonated malt beer (Les Trois Brasseurs), protein content: 0.9 g/l, various amounts of a prolyl-specific endoprotease enzyme ("Endo-Pro A", see Materials) were added. Two series of haze-measurements were performed. In the first series, the beer-Endo-Pro A compositions were incubated at 40°C for 1 hour prior to the Chill haze test. After incubation at 40°C, and just prior to the Chill haze test, ethanol was added to the beer-Endo-Pro A composition in an amount sufficient to increase the alcohol content to 6% (v/v). In the second series, the beer without Endo-Pro A was incubated at 40°C for 1 hour, and then cooled to 0°C. At 0°C, the Endo-Pro A was added and the resulting compositions were incubated at 0°C for 15 min. Just prior to the Chill haze test, ethanol was added to the beer-Endo-Pro A composition in an amount sufficient to increase the alcohol content to 6% (v/v).

The amounts of Endo-Pro A added and the percentage of haze reduction relative to the haze measured when no Endo-Pro A was added are shown in Table 1. The amounts of Endo-Pro A added covered a large range from less than 1% of exogenous proteins added to more than 10 %, relative to the amount of protein present in the beer.

Table 1. Effect of the addition of a prolyl-specific endoprotease enzyme to a beer on

the amount of haze after incubation at 40°C for 1 h

Trial	"Endo-Pro A" added		Chill Haze test (EBC)	Haze reduction (%)
	µl/10ml of beer	% of exogeneous protein added*		
1	0	0	141	0
2	0.45	0.25	116	17.7
3	0.9	0.5	101	28.4
4	1.8	1	87.4	38.0
5	3.6	2	81.1	42.5
6	5.4	3	69.0	51.1
7	9	5	61.3	56.5
8	18	10	48.5	65.6
9	36	20	39.9	71.7
10	54	30	33.4	76.3

\* "% of exogenous enzyme added" reflects the amount of Endo-Pro A"-enzyme added expressed as a percentage of the total amount of proteins present in the beer before addition of the enzyme.

5

Table 2. Effect of prolyl-specific endoprotease enzyme to a beer on the amount of haze after incubation at 0°C for 15 min

Trial	Endo-Pro A added		Chill Haze test (EBC)	Haze reduction (%)
	µl/10ml of beer	% of exogeneous protein added		
1	0	0	141	0
2	0.45	0.25	141	0
3	0.9	0.5	141	0
4	1.8	1	143	-1.4
5	3.6	2	145	-2.8
6	5.4	3	138	2.1
7	9	5	138	2.1
8	18	10	130	7.8
9	36	20	126	10.6
10	54	30	120	14.9

Table 1 clearly illustrates that less haze is formed upon chilling when a prolyl-specific endoprotease is added to beer at a temperature when the protease is active prior to chilling. Table 2 clearly illustrates that there is some effect when a prolyl-specific endoprotease is added to the beer at a temperature so low that the protease is not or hardly active, but the effect is very small in comparison to the effect observed when the protease is added at a temperature where it is active.

#### Example 4

#### 10 Effects of the addition of Papain on haze formation in beer

To a decarbonated malt beer (Les Trois Brasseur), protein content: 0.9 g/l, various amounts of papain (from 0 to 100 g/hl) were added. Two series of chill haze-measurements were performed. For the first series, the beer-papain compositions were incubated at 40°C for 1 hour prior to the Chill haze test. Ethanol was added to the incubated samples to reach 6% alcohol (v/v) prior to the haze measurements. In the second series, beer samples were incubated at 40°C for 1 hour and subsequently cooled to 0°C. Then, papain was added and the samples were incubated at 0°C for 15 min. The amounts of papain added and the percentage of haze reduction relative to the haze measured when no papain was added are shown in Table 3.

Table 3. Effect of papain on the amount of haze formed in beer after incubation at 40°C for 1 h

Trial	Papain added		Chill Haze test (EBC)	Haze reduction (%) (%)
	g/hl beer	% of exogeneous protein added		
1	0	0	143	0
2	0.2	0.03	140	2.1
3	0.5	0.07	133	7.0
4	1	0.13	119	16.8
5	2	0.26	94.1	34.2
6	3 <sup>(1)</sup>	0.40	91.6	35.9
7	5	0.66	83.8	41.4
8	8	1.06	82.4	42.4
9	10	1.32	84.0	41.3
10	100	13.22	81.6	42.9



(1) 3 g/hl is the maximal dosage recommended

Table 4. Effect of papain on the amount of haze after incubation at 0°C for 15 min

Trial	Papain added		Chill Haze test (EBC)C	Haze reduction (%)
	g/hl beer	% of exogenous protein added		
1	0	0	143	0
2	0.2	0.03	139	2.8
3	0.5	0.07	136	4.9
4	1	0.13	135	5.6
5	2	0.26	134	6.3
6	3	0.40	132	7.7
7	5	0.66	136	4.9
8	8	1.06	130	9.1
9	10	1.32	122	14.7
10	100	13.22	138	3.5

The results in Table 3 illustrate the effect of papain on the amount of haze formed in beer upon chilling. It is clear that the effect of papain on haze levels off when papain is added in an amount of 3 g/hl and higher. Apparently, it is not possible to achieve the same amount of haze reduction with papain as with a prolyl-specific endoprotease.

#### Example 5

#### Effects of the addition of PVPP on haze formation in beer

In both beer-prolyl-specific endoprotease experiments and the beer-papain experiments, a control experiment was done by adding a large amount of PVPP (1000 g/hl) to the beer prior to incubation. After 15 min of mixing the PVPP was removed by filtration (No prolyl-specific endoprotease enzyme or papain was added). In both controls almost no haze were formed during the chill haze test. Since it is known that PVPP removes polyphenols from beverages, these control experiments indicate that polyphenols do take part in haze formation in beer. To measure the PVPP effect on beer haze stability, different amounts of PVPP were added to a decarbonated beer and removed by filtration after 15 min of mixing. Prior to adding the PVPP the beer was incubated during 1h at 40°C.

Table 5 shows the effect of the addition of various amounts of PVPP

on the amount of haze present in beer upon chilling. No Endo-Pro A or papain was added.

Table 5. Effect of PVPP on the amount of haze present in beer upon chilling

PVPP added (g/hl)	Chill Haze test (EBC)	Haze reduction (%)
0	133	0
10	131	1.5
20	115	13.5
30 <sup>(1)</sup>	103	22.6
50	65.2	51.0
80	48.4	63.6
100	42.1	68.3
500	17.2	87.1
1000	9.5	92.9

30g/hl maximal dosage used in breweries

5

In Table 3 it is shown that the addition of 3g of Papain / hl of beer (which is the maximal dosage recommended in the beer industry) after incubation at 40°C for 1 hour induces an haze decrease of almost 36%. In the case of addition of the prolyl-specific endoprotease, the addition of 1% (relative to the amount of protein in the beer) of prolyl-specific endoprotease after incubation at 40°C for 1 hour induces a decrease of a beer chill haze of 38% (see Table 1). In breweries, PVPP is generally added in a quantity that does not exceed 30 g/hl. Since PVPP reduces haze by around 20% when it is added in that quantity, it can be concluded that both papain and prolyl-specific endoprotease enzymes are better haze inhibitors than PVPP.

10

15

#### Example 6

Endo-Pro A addition on a 100% malt mash and haze reduction in a 100% malt wort

The objective was to determine if the addition of Endo-Pro A to a 100% malt mash could result in an haze reduction in the final malt wort.

20

Each mashing trial begins with the mixing of 25 g of milled malt with 100 ml of water. Then, the mash is heated to 50°C and after the addition of an amount of "Endo-Pro A" the mash is treated according to a step-wise heating procedure to four successively higher temperatures. Table 6 shows that mashing temperature profile.

During all the mashing the mash was stirred at 200 rpm. At the end of the mashing, the

25

mash is kept at room temperature and water was added to compensate the water evaporation. Then, the mash was filtered on paper to separate the wort (liquid) from the solids.

Table 6. Mashing temperature profile

Steps	Temperature	Time
1	50°C	30 min
temperature increase	1°C/min	13 min
2	63°C	30 min
temperature increase	1°C/min	10 min
3	72°C	30 min
temperature increase	1°C/min	4 min
4	77°C	10 min

5

0, 200 & 500 µl Endo-Pro A was added to the mashes, respectively.

A control experiment was performed wherein 500µl of Endo-Pro A was used that was deactivated by heating it to 90°C during 15 min. The turbidity or haze of the wort was measured at room temperature and after a chill haze test. Wort chill tests are performed as described in the Alcohol/low-temperature test according to Chapon (Chill Haze test –Pfeuffer Operating instructions) adding ethanol to reach 10% (v/v) in the sample as recommended by Chapon for alcohol-free beers.

10

Table 7: effect of the addition of prolyl-specific endoprotease to 100% malt mashes on the amount of haze formed in the resulting 100% malt worts (after Chill haze test)

15

Trial	Endo-Pro A added in the mash (µl)	Wort initial turbidity (EBC <sup>(1)</sup> )	Wort turbidity after Chill Haze test (EBC)	Haze induced by the Chill Haze test (ΔEBC)	Endo-Pro A haze reduction effect (%)
1	0	10	158.5	148.5	0
2	200	15.9	59.2	43.3	70.8
3	500	22.5	49.1	26.6	82.1
4	500 (desactivated)	12.1	160.5	148.4	0.1

EBC: nephelometric turbidity units recommended by the European Brewery Convention

The results in Table 7 clearly indicate that when a prolyl-specific endoprotease has been added to a malt mash, the resulting wort is much less turbid upon cooling than a wort prepared without the addition of a prolyl-specific endoprotease.

5 To study the Endo-Pro A effect, a chill haze test was performed on malt wort. It was observed that the addition of a prolyl-specific endoprotease decreased the wort chill haze. A decrease in haze formed in the Chill haze test was observed even at low quantities of prolyl-specific endoprotease enzyme added. When the enzyme is deactivated prior to be added in the mash, the stabilization effect disappears  
10 completely i.e. the amount of haze formed is no longer reduced. The haze decrease induced by the addition of a prolyl-specific enzyme is very important. In the Example a haze reduction of up to 82 % was achieved.

In order to compare the effects of the addition of a prolyl-specific endoprotease enzyme in malt worts and in barley worts, experiments were performed  
15 wherein different amounts of Endo-Pro A were added in barley mashes. The pH were respectively 5.6 in malt worts and 6.1 in barley worts. The results obtained with barley worts chill haze tests showed that as observed previously for malt worts, the treatment of barley mashes by a prolyl-specific endoprotease induces an important reduction of barley worts chill haze. Both malt and barley mashes treated with a prolyl-specific  
20 endoprotease result in highly stabilized worts but the effect is stronger in malt worts than in barley worts. Indeed, the addition in the mash of 200µl of Endo-Pro A induced a haze reduction of around 59% in barley worts and more than 70% in malt worts. The trials performed with 500µl of Endo-Pro A increased the haze reduction until 82% in malt worts and did not improve the haze reduction in barley worts compared to the  
25 200µl Endo-Pro A experiment. Surprisingly, the addition of Endo-Pro A to barley mashes resulted in clear filtered worts while the non-Endo-Pro treated mashes resulted in cloudy filtered worts (the barley mash filtrations were performed at room temperature and the turbidity of the worts was measured at room temperature without ethanol addition). That effect is observed whatever the amount of prolyl-specific endoprotease  
30 added in the barley mashes.

### Example 7

#### Haze reduction in wine

35 Different dosages (0, 30, 60 150 µl) of a prolyl-specific endoprotease (Endo-Pro B) having a specific activity of 6.0 U/ml were added to flasks containing 500

ml of white wine (wine as described under "Materials") and incubated at room temperature (22-25°C) for 19 days under a nitrogen atmosphere. The wine haze stability was measured after 0, 6, 8, 12 and 19 days using the Heating test as described under "Methods".

5                      The results of the experiments are shown in Table 8. In Table 8, wine turbidity or haze is expressed in nephelos turbidity units (NTU).  $\Delta$ NTU = turbidity in NTU measured on wine samples after heating - turbidity in NTU measured on wine samples before heating. The quantity of bentonite required to stabilize the proteins of the wine was calculated according to the formula:  $(1.48 \times \Delta \text{NTU}) + 2$ . As is known, less  
10 bentonite is needed to prevent haze formation in wine when a wine is less susceptible to haze formation.

Table 8. Effect of the addition of a prolyl-specific endoprotease enzyme to a white wine the amount of haze formed after heating the wine

15

Incubation time	Endo-Pro added ( $\mu$ l/500ml)	Turbidity before heating (NTU)	Turbidity after heating (NTU)	$\Delta$ NTU	quantity of bentonite required (g/hl)	haze reduction (%)	Decrease of the quantity of bentonite required (%)
0	0 $\mu$ l (Control)	36.8	74.5	38	58		
6 days	0 $\mu$ l (Control)	25.8	63.8	38	58	0	0
	30 $\mu$ l	20.2	53.5	33	51	13	12
	60 $\mu$ l	21.6	56	34	53	11	9
	150 $\mu$ l	20.5	43.3	23	37	39	36
8 days	0 $\mu$ l (Control)	26.4	68	42	64	0	0
	30 $\mu$ l	31.4	60.2	29	45	31	30
	60 $\mu$ l	29.6	56.2	27	41	36	36
	150 $\mu$ l	25.1	48.4	23	36	45	44
12 days	0 $\mu$ l (Control)	24.4	50.1	26	40	0	0
	30 $\mu$ l	23	36.9	14	23	46	43
	60 $\mu$ l	23	36.6	14	23	46	43
	150 $\mu$ l	23.9	37.9	14	23	46	43
19 days	0 $\mu$ l (Control)	6.5	23.2	17	26	0	0
	30 $\mu$ l	5.8	11.2	5	10	71	62
	60 $\mu$ l	7	11.1	4	8	76	69
	150 $\mu$ l	8.5	13.7	5	10	71	62

The results in Table 8 show that the addition of a prolyl-specific endoprotease to a white wine before heating reduces the haze formed in the wine after heating. After 6 days of incubation at room temperature the effect is observed. Indeed, the haze decrease reaches 39% with 150  $\mu$ l of the Endo-Pro B- added and around 12% with 30  $\mu$ l or 60  $\mu$ l of Endo-Pro B. After 12 days and whatever the amount of the

prolyl-specific endoprotease used the haze reduction reaches 46% and exceeds 70% after 19 days. Therefore, it is clear that a prolyl-specific endoprotease can be used to avoid or to strongly reduce the quantity of bentonite required to stabilize wine against haze formation.

5

### Example 8

#### Haze reduction in Strawberry Juice

A strawberry fruit juice was prepared as follows: strawberries were  
10 defrosted and crushed, and subsequently blanched (heated) at 90°C in order to destroy all endogenous enzymes such as polyphenol oxidases, and to denature proteins. The crushed strawberries were then cooled to 50°C, macerated for 30 min at 50°C with 600g/t of Rapidase BE super (a commercial enzyme product of DSM, France) and pressed in a pneumatic press. In order to remove the denatured proteins the resulting  
15 mixture was centrifuged at a speed of 8000 rpm and filtered. The strawberry juice was collected. An acidified alcohol test was negative, which confirmed that the juice was pectin free. The pH value of the juice was 3.3.

Endo-Pro A/ strawberry juice incubations: Different volumes (0, 5, 10, 20 µl) of Endo-Pro A (5.06 U/ml) were added to 100 ml of strawberry juice and  
20 incubated at 50°C for 60 min. Two control experiments were performed, (i) one by adding 20µl of deactivated Endo-Pro A (incubated 30 min at 80°C) and (ii) a second control by adding 200 mg of PVPP to 20 ml of strawberry juice previously incubated 1 h at 50°C. After having been mixed for 15 min at room temperature the PVPP was removed by centrifugation.

25 Juice Heating test: the juice turbidity was measured before and after heating the fruit juice samples at 80°C during 30 minutes. Before measuring the turbidity, the heated juices were cooled down under cold water.

The turbidity measurements were performed in a turbidimeter previously calibrated with NTU turbidity standards from Reagecon (Ireland)

30

Table 9: Effect of the addition of prolyl-specific endoprotease on the haze in strawberry juice

Trial	Endo-ProA added ( $\mu$ l/100ml)	Turbidity before heating (NTU)	Turbidity after heating (NTU)	$\Delta$ NTU	reduction effect (%)
1	0	10.1	17.5	7.4	0
2	5	9.5	15.0	5.5	25.7
3	10	9.4	15.4	6.0	18.9
5	10 (deactivated)	10.2	16.9	6.7	9.4
6	PVPP	1.4	2.3	0.9	

5 The results in Table 9 show that the addition of 5  $\mu$ l of Endo-Pro A in 100ml strawberry juice decreased haze formed after a juice heating test by 25.7%. The addition of 10 $\mu$ l of Endo-Pro A to 100ml strawberry juice did not improve the haze reduction effect compared to the 5 $\mu$ l trial. Possibly, the enzyme action is maximal with 5 $\mu$ l and with more enzyme addition more protein precipitation is obtained.

10 Deactivated enzyme still reduced the amount of haze formed, however, the effect was much less pronounced. After the addition of PVPP, hardly any haze was observed, but the color of the sample was also removed. The fact that the addition of PVPP results prevents haze formation indicates that also in strawberry juice haze is probably the result of polyphenol-protein interactions.

15

### Example 9

#### Isolation of a proline-specific carboxypeptidase from *Xanthomonas* species

20 Although many scientific reports describe exopeptidases that are able to act on proline residues at the carboxyterminal ends of peptide chains, the hydrolysis rate of these carboxypeptidases towards proline residues is very low. Moreover most of these carboxypeptidases are not capable of releasing carboxyterminal proline residues from polyproline sequences. US 5,693,503 describes the purification of carboxypeptidases from *Xanthomonas* species that exhibit a surprisingly high activity towards the release  
25 of carboxyterminal proline residues, even from polyproline sequences. We have



isolated such a carboxypeptidase from several *Xanthomonas* species to test their activity in preventing haze formation.

The isolation of the desired carboxypeptidase from *Xanthomonas* proved to be a difficult task since the purification method as described in US 5,693,503 was not successful in our hands and a completely new purification protocol had to be developed. Of the many procedures tested the following protocol yielded substantially purified enzyme.

Starting from one liter of broth, cells were harvested by centrifugation, washed once with water and then disrupted by an ultrasonic treatment at 0 degrees C.

Cell debris was removed by a centrifugation at 20000 rpm for 20 minutes and the resulting supernatant was recentrifuged under the same conditions. Then ammonium sulfate was added to the supernatant to reach 40% saturation. After low speed centrifugation the supernatant was collected and additional ammonium sulfate was added to reach 80% saturation. The newly formed precipitate was collected by low speed centrifugation, extensively dialysed at pH 7.0, 4 degrees C and applied to a bacitracin-Sepharose column equilibrated in 0.05 mol/liter of tris-HCl pH 8.0. (J. Appl. Biochem., 1983 pp420-428). Material bound to the column was eluted using the equilibration buffer supplemented with 1 mol/liter of NaCl and 10% (v/v) isopropanol. The fractions containing the enzyme activity sought were identified by incubation with the synthetic substrate Z-Pro-Pro as described in the Materials & Methods section. Fractions containing activity were then applied to a MonoQ column equilibrated in 0.025 mol/liter Tris-HCl pH 8.5. Under these conditions the enzyme bound to the column and was eluted using an NaCl concentration gradient. Active fraction were pooled, dialysed and rechromatographed on a Mono Q column as described. The activity of the final concentrate measured on Z-Pro-Pro at 37 degrees C and pH 7.0 was 0.5 units/ml and 0.09 units/ml at pH 5.5. The enzyme preserved approx 50% of its activity after a 2-3 hours incubation at 50 degrees C at pH 5.5. This preparation has been used in the tests described in Example 11.

Only the enzyme isolated from *Xanthomonas campestris* pv *campestris* showed activity under acid conditions. A similar carboxypeptidase isolated from *X.rubrilineans* showed no activity at pH 5.5 and is, therefore, less suited for the applications anticipated.

**Example 10**Isolation of a glycine-cleaving endoprotease from *Aspergillus oryzae*

5 An endoprotease that can cleave proteins rich in glycine residues has been identified in papaya extracts (glycyl endopeptidase, EC 3.4.22.25). However, this enzyme has several disadvantages for the application as envisaged because of its relatively high (near neutral) pH optimum and high costs if produced in a pure form. Therefore the identification of an acid stable, glycine-specific endoprotease the enzyme in a potentially  
10 foodgrade microorganism could offer many advantages. To that end we have screened a number of commercially available foodgrade enzyme preparations.

The screening was carried out using the synthetic chromogenic peptide Z-Gly-Gly-pNA at pH 4.0 as the substrate. Six different enzyme preparations were tested and only  
15 Flavourzyme 1000 L (HPN 00011 from *Aspergillus oryzae*; NOVO, Denmark) showed some activity towards the synthetic substrate used. To isolate the enzyme responsible for this activity a large number of different purification protocols had to be tested. The following protocol proved to be successful.

First, the excess of small molecular weight components as present in the Flavourzyme  
20 material was removed by an extensive dialysis. During this dialysis step the Gly-specific activity dropped till approx 50% of its initial value. The resulting dialysed liquid was then further washed and concentrated using Amicon PM-10 ultrafiltration equipment. To the resulting liquid concentrate ammonium sulfate was added till 50% and after centrifugation more ammonium sulfate was added to the supernatant to reach  
25 75% of the saturation level. The precipitate then obtained was collected by low speed centrifugation, dialysed and the resulting liquid was again concentrated using the PM-10 equipment. The concentrate was applied on a Sephadex G-100 gelfiltration column and the active fractions were pooled. The activity in each fraction was measured using Z-Gly-Gly-pNA at pH 4.0 as described in the Materials & Methods section. The pooled  
30 fractions were then subjected to ion exchange chromatography on H1Trap Q equilibrated in 0.05 mol/liter sodium acetate pH 5.0 and eluted with the same buffer containing 1 M NaCl. As before active fractions were identified by incubation with Z-Gly-Gly-pNA but now at pH 5.0, pooled and concentrated using the PM-10 equipment. The final concentrate if measured at pH 5.0 contained 0.008 units/ml. This solution was  
35 used to carry out the experiments as described in Example 11.

**Example 11**Improved haze stability of 100% malt worts treated with selected auxillary proteases

- 5 The present Example demonstrates that the anti-haze effect of Endo-ProA can be further improved by combining the Endo-ProA treatment with selected auxillary proteases. It is exemplified that a proline-specific carboxypeptidase from *Xanthomonas campestris* ("CarboxyPro") or a glycine-cleaving endoprotease from *Aspergillus oryzae* ("EndoGly") significantly reduce haze formation further when used in combination with
- 10 Endo-ProA. The present Example focuses on haze reducing effects in 100% malt worts.

To prepare malt wort, 50 g of milled malt was mixed with 200 ml of water and heated to 50°C. Either 0 or 150µl of the Endo-Pro enzyme concentrate was added and the mash was subjected to a step-wise heating protocol as specified in Table 10. During this

15 procedure the mash was stirred at 200 rpm. At the end, the mash was cooled to room temperature and water was added to compensate for any water evaporated. Finally the mash was filtered on paper to separate the wort (liquid) from the solids. The worts thus obtained were used to test the effects of the auxillary enzymes using the effect of PVPP as a reference.

20

Table 10. Mashing temperature protocol

Steps	Temperature	time
1	50°C	30 min
temperature increase	1°C/min	13 min
2	63°C	30 min
temperature increase	1°C/min	10 min
3	72°C	30 min
temperature increase	1°C/min	4 min
4	77°C	10 min

- Because of its high affinity for polyphenol, PVPP is widely used in the industry to remove beer hazes caused by polyphenol-protein aggregates. In order to determine if
- 25 CarboxyPro or EndoGly can have a haze reducing effect as PVPP, non-Endo-Pro treated worts were incubated with either 100µl CarboxyPro or 20µl EndoGly during 120.

minutes at 50 degrees C after which the resulting haze stabilities were measured according to the procedure described in the Materials & Methods section. As a reference, non-Endo-Pro treated mash was incubated with PVPP for 15 minutes at room temperature prior to filtration and then measured. The results obtained are shown in Table 11.

Table 11: The chill haze effects of PVPP, CarboxyPro and EndoGly on a non-Endo-Pro treated 100% malt wort.

	Enzyme active conditions			Controls	
TRIALS n°	1	2	3	4	5
Enzyme added in the mash					
Endo-Pro (µl)	0	0	0	0	0
Enzymes added in the wort	120 min at 50°C				
Proline Carboxypeptidase (µl)	0	100	0	0	0
EndoGly (µl)	0	0	20	0	0
Wort haze control				PVPP is mixed 15 min prior paper filtration	
PVPP (Control - g/hl)				30	100
Haze measurement					
Wort Chill Haze test (EBC)	146	131	91.7	75.3	69.8
	144	134	92.7	79.0	68.1
Haze induced by the Chill Haze test (average - EBC)	145	132.5	92.2	77.2	69.0
Haze reduction (%)	0.0	8.6	36.4	46.8	52.4

10 EBC: nephelometric turbidity units recommended by the European Brewery Convention

According to the results shown in Table 11, incubations with both CarboxyPro and EndoGly result in worts that are less turbid upon cooling. However, the results also indicate that the addition of PVPP has a clearly superior effect.

15 A subsequent experiment was carried out in which a wort was used

that had been pretreated with Endo-Pro in the mashing stage. In this experiment, the auxillary proteases were added to the wort in quantities of either 0, 100 or 500 microliters EndoGly or 0, 20 or 100 microliters CarboxyPro per 10 millilitres wort. All samples were then incubated for 120 min at 50°C after which the chill haze test was carried out. The results obtained are provided in Table 12.

Table 12: Wort chill haze results obtained after incubation of an Endo-Pro A treated 100% malt wort followed by incubation with various amounts of CarboxyPro or EndoGly .

TRIALS n°	Enzyme active conditions					
	1	2	3	4	5	6
Enzyme added in the mash						
Endo-Pro (µl)	0	150	150	150	150	150
Enzymes added in the wort	120 min at 50°C					
Proline Carboxypeptidase (µl)	0	0	100	500	0	0
EndoGly (µl)	0	0	0	0	20	100
Haze measurement						
Wort Chill Haze test (EBC)	168	79.2	68.8	58.8	63.0	40.1
Haze induced by the Chill Haze test (average - EBC)	168	80.6	70.0	57.7	64.8	36.0
Haze reduction (%)	168	79.9	69.4	58.3	63.9	38.1
Enzymes effect on Chill Haze Haze reduction (%)	0.0	52.4	58.7	65.3	62.0	77.4
CarboxyPro or EndoGly effect on Endo-Pro treated wort Haze reduction (%)		0.0	13.1	27.1	20.0	52.4

EBC: nephelometric turbidity units recommended by the European Brewery Convention

According to the results shown in Table 12, the combination of an Endo-Pro pretreatment with either CarboxyPro or EndoGly have significant effects on the haze stability of a wort. Depending upon the concentration of the auxillary proteases added effects can be obtained that are superior those obtained with PVPP in non-Endo-Pro

treated wort (see Table 11).

- 5 Finally an experiment was carried out to test if the enzyme effects observed are the result of non-enzymic artefacts or specific proteolytic action. To that end 100 µl CarboxyPro or 20µl EndoGly were added to an Endo-Pro-treated wort. To prevent all enzymatic activities of the auxillary enzymes, the wort containing these enzymes was kept at 0 degrees C for 15 minutes (i.e. comparable with the incubation period used for PVPP) before measuring the wort chill haze formed. Wort treated with various quantities of PVPP and then filtered was used as a reference.

Table 13: Effects of the incubation of Endo-Pro-treated 100% malt wort with CarboxyPro or EndoGly at 0 degrees C , or PVPP at room temperature

TRIALS n°	Controls				
	1	2	3	4	5
Enzyme added in the mash					
Endo-Pro A (µl)	150	150	150	150	150
Enzymes added in the wort	enzymes added in non-active condition (at 0°C after the Endo-Pro treated wort was incubated 120 min at 50°C)				
Proline Carboxypeptidase (µl)	100	0	0	0	0
EndoGly (µl)	0	20	0	0	0
Wort haze control			PVPP is mixed 15 min with Endo-Pro treated wort previously incubated 120 min at 50°C. PVPP removal is performed by paper filtration		
PVPP (Control - g/hl)			30	100	500
Haze measurement					
Wort Chill Haze test (EBC)	77.5	82.2	66.3	59.3	52.3
Haze induced by the Chill Haze test (average - EBC)	77.5	82.2	65.9	60.6	53.5
Enzymes effect on Chill Haze Haze reduction (%)	53.9	51.1	66.1	60.0	52.9
Haze reduction (%)	3.0	-2.9	60.7	64.3	68.5
			17.3	25.0	33.8

EBC: nephelometric turbidity units recommended by the European Brewery Convention

The data presented in Table 13 show that both CarboxyPro and EndoGly if incubated at 0 degrees have no effect which implies that their proteolytic activities are the direct cause for haze stabilisation observed in Tables 11 and 12. PVPP has a limited effect only (a haze reduction of a mere 17.3% versus a haze reduction of 46.8% under conditions described in Table 11). This observation re-confirms the impact an Endo-Pro incubation has on the total quantity of precipitable polyphenol-protein present: by preventing the formation of polyphenol-protein aggregates, the use of PVPP has almost become superfluous.

### Example 12

#### Endo-Pro A has also Endo-Hydroxy-Pro and Endo-Ala activity

It was investigated whether Endo-Pro A also has endoprotease specificity for hydroxy proline and alanine. Therefore three synthetic peptides were synthesized covering the C-terminus of  $\alpha_{s1}$ -caseine B, with some modifications. The peptides were:

- 0139-59, SEKTTMPLW, the original C-terminus of  $\alpha_{s1}$ -caseine M=1091.5
- 0139-60, SEKTTMJLW, with J being hydroxy proline M=1107.5
- 0139-61, SEKTTMALW, with alanine substituted for proline M=1065.5

For analyzing such complex peptide mixtures so called *scan dependent MS/MS* is used. This method, in which each scan consists of three segments, is defined as follows:

- 1: *full scan* analysis,
- 2: *zoomscan* analysis for the determination of the charge state of the most intense ion in the full scan mass range,
- 3: *MS/MS* of the most intense ion in the full scan mass range to obtain amino acid sequence information.

All three peptides were dissolved in 0.1 % formic acid at a concentration of 75-100  $\mu\text{g/l}$  and were checked for their purity in LC/MS and LC/MS/MS mode using gradient elution. All three peptides could be identified by their protonated and doubly protonated molecules in LC/MS mode and in LC/MS/MS mode by total coverage of the amino acid sequence.



The synthetic peptides treated with Endo-ProA were diluted 50 times before analysis. For LC/MS analysis of the peptides formed after treatment, the gradient has to be adapted slightly. If indeed Endo-ProA has proline and hydroxy proline specific specificity the peptides should be cleaved in the following parts:

5

SEKTTMP and LW, with protonated molecules at  $m/z$  793.4 and 318.1, respectively.  
SEKTTMJ and LW, with protonated molecules at  $m/z$  809.4, and 318.1, respectively.

Both predicted peptide masses could indeed be observed in the ion chromatograms of  
10 the Endo-Pro treated peptides, and were checked in LC/MS/MS mode having the correct amino acid sequence.

The third synthetic peptide also showed the same pattern by  
observing  $m/z$  767.4 of SEKTTMA and  $m/z$  318.1 of LW in the ion chromatogram.  
Endo-Pro preferentially cleaves C-terminal at P, J and A, but also peptides being 1,2,  
15 or 3 amino acids shorter could be observed and unequivocally be identified with LC/MS/MS. However these percentages were all below 6 %.  
An overview of the above is given in table 14.

Table 14: peptide formed for all three synthetic peptides after treatment with Endo-Pro  
20 A. Amino acid sequences were checked by their protonated molecule and MS/MS characteristic.

peptide formed	intensity normalized		
	SEKTTMPLW	SEKTTMJLW	SEKTTMALW
SEKT		0,43	0,33
SEKTT	0,00	0,37	0,33
SEKTTM	3,40	3,11	5,56
SEKTTMP	100,00		
SEKTTMJ		100,00	
SEKTTMA			100,00

## 25 Conclusion

Indeed Endo-Pro A can cleave hydroxy proline and alanine residues at their carboxyterminal side in addition to its preference for cleaving proline..

**Example 13**

PH optimum for Endo-Pro.

5 DNA sequences according to SEQ ID NO: 1 and SEQ ID NO: 3 were expressed in  
Aspergillus niger iso 502. This resulted in polypeptides according to SEQ ID NO: 5 and  
SEQ ID NO: 4 respectively. These polypeptides will be further referred to as Endo-Pro  
RUS and Endo-Pro GAM respectively. Both enzymes were found to be very much alike  
but were also found to differ in some biological aspects. Endo-Pro RUS had a higher  
10 specific activity and was found somewhat more active in removing haze in beer.

**1) pH optimum Endo-Pro:**

Proteolytic activity of Endo-Pro's were measured using resorufin-labeled  
casein and the prescription of Roche ("Universal Protease Substrate"; Cat. No 1  
080 733). By treatment with proteases, resorufin-labeled peptided are released  
15 which cannot be precipitated with trichloroacetic acid. So the more active the Endo-  
Pro enzyme is at a given temperature or pH value, the higher the light absorption at  
574 nm. in a pH range of pH 4.5 – 4.8 – 5.0 – 5.5 – 6.0 – 7.0 – 8.0. 0.1 M Tris/HCl  
buffers were used for pH 7 and 8. For the lower pH's 0.1 M Hac buffers were used.  
The buffers contained 0.02 M CaCl<sub>2</sub>.

20 Reactions were performed for 30 minutes at 37 °C and the data obtained  
are provided in Table 15 Endo-ProRUS (6 U/mL) and Endo-ProGAM (ca. 4 U/mL)  
were diluted to 0.5 U/mL. 10 µL sample was added in the incubation mixture and  
volume compensated with the incubation buffer.

According to the results shown in Table 15 the pH optimum of the enzymes  
25 is around pH 5.5. However, later on it became clear that the resorufin-labeled  
casein used starts to precipitate at pH values below pH 5.5 so that below this pH  
unreliable results are obtained. To correct for that, the experiment was repeated  
under the various pH conditions mentioned but using Z-Gly-Pro-pNA rather than  
resorufine-labeled casein as the chromogenic substrate. The data obtained using  
30 Z-Gly-Pro-pNA and a light absorption at 410nm (cf Example 2) showed a clear  
activity peak around pH 4.5 for both Endo-Pro enzymes.

**2) Temperature stability of Endo-Pro enzymes.**

The temperature stability of both Endo-Pro enzymes was measured by heating the two enzymes to temperatures of either 40 – 50 – 55 and 60 °C and at pH 5.0. Samples were taken after 0.5 – 1 – 2 – 5 and 20 hours and the Endo-ProRUS and Endo-ProGAM activities were diluted to approx 0.5 U/mL. Then 10 µL sample was added in the incubation mixture containing Z-Gly-Pro-pNA (cf Example 2) and residual enzyme activities were measured as described in Example 2. In the reference the 10 microliter volume was compensated with the incubation buffer. The Z-Gly-Pro-pNA cleaving activity of the non-heated enzyme was used as the 100% value.

From the results shown in Table 16 it is clear that both Endo-Pro enzymes show excellent temperature stabilities.

**Table 15 - pH optima Endo-Pro enzymes**

pH	RUS		GAM	
	E <sub>574</sub>	Rel. %	E <sub>574</sub>	Rel. %
4.5	0.227	30.2	0.225	24.0
4.8	0.228	38.3	0.278	29.6
5.0	0.521	69.4	0.554	59.0
5.5	0.751	100.0	0.939	100.0
6.0	0.675	89.9	0.870	92.7
7.0	0.399	53.1	0.627	66.8
8.0	0.011	1.5	0.313	33.3

**Table 16 Temperature- stabilities Endo-Pro enzymes**

t [hours]	RUS				GAM			
	4	5	5	6	4	5	5	6
	0 °C	0 °C	5 °C	0 °C	0 °C	0 °C	5 °C	0 °C
0	100	100	100	100	100	100	100	100
0.5	93	102	100	67	98	94	94	84
1	105	98	98	81	102	97	92	83
2	99	102	96	74	103	95	94	79
5	99	99	88	67	95	88	92	80
22	104	90	66	33	105	88	82	64

**Example 14****5 Haze reduction under beer fermentation and lagering conditions.**

In previous Examples we have demonstrated the efficacy of enzymatic haze prevention at both room temperatures (cf Example 7) and at elevated temperatures. In the present Example we illustrate the versatility of this enzymatic Endo-Pro approach by showing that proline-specific endoproteases can also effectively prevent haze formation under conditions where it has to be active under conditions which are far from optimal for the enzyme. To that end we have tested whether the Endo-Pro enzyme can prevent haze formation in beer if added prior to beer fermentation (so that the enzyme can work during 10 days at 12 degrees C) or if added prior to beer lagering (so that the enzyme can work during 10 days at 4 degrees C).

To test the enzymatic haze preventing effect during beer fermentation, we have used a freshly produced, 100% malt beer containing approx 4% ethanol that had been membrane filtered but did not undergo any treatment to remove haze forming components. This "non-stabilized" beer was decarbonated after which various concentrations of Endo-Pro enzyme were added (see Table 17). This mixture was then

incubated at 12°C during 10 days to mimic an industrial beer fermentation as closely as possible.

To test the enzymatic haze preventing effect during lagering conditions, exactly the same starting material was used in combination with the same concentrations of the

- 5 Endo-Pro enzyme, but now the mixture was incubated at 4 degrees C during 10 days.

In both experiments PVPP ((Polyclar AT / water un-soluble) dosages of 20, 30 and 50 grams per hectoliter of beer were used (agitated during 15 min at room temperature prior a paper filtration) as a reference.

- 10 In both experiments, papain (liquid DSM Collupulin; batch no 010604, the final activity of 5480 NFU/mg) was also included in dosages of 1, 2 and 3 grams per hectoliter.

In all samples haze formation was measured as specified in Example 2.

**Table 17: Enzymatic haze prevention during fermentation**

	Decarbonated, non-stabilized, 100% malt beer											
Trial	1	2	3	4	5	6	7	8	9	10	11	12
Incubation	Imitating fermentations conditions (12°C for 10 days)											
<b>Additions</b>												
Endo-Pro (U/l of beer)	-	-	0.5	1	2	4	-	-	-	-	-	-
Papain (g/hl of beer)	-	-	-	-	-	-	1	2	3	-	-	-
PVPP (g/hl of beer)	-	-	-	-	-	-	-	-	-	20	30	50
<b>Haze</b>												
Chill Haze test (EBC)	170	173	35	30	24	23	95	102	101	124	120	105
	173	173	44	31	22	23	95	102	97	128	121	108
Average	172	173	40	31	23	23	95	102	99	126	121	107
Chill Haze reduction effect (%)	-	-	77	82	87	87	45	41	43	27	30	38
EBC Haze test 60° t=0	9	9	5	4	4	4	8	10	11	5	4	4
EBC Haze test 60° t=48H	55	56	29	27	27	38	58	63	74	24	19	13
Chill Haze reduction effect (%)	-	-	48	52	52	31	-5	-13	-32	58	66	77

**Table 18: Enzymatic haze prevention during lagering**

Trial	Decarbonated, non-stabilized, 100% malt beer											
	1	2	3	4	5	6	7	8	9	10	11	12
Incubation	Imitating beer lagering (4°C for 10 days)											
<b>Additions</b>												
Endo-Pro (U/l of beer)	-	-	0.5	1	2	4	-	-	-	-	-	-
Papain (g/hl of beer)	-	-	-	-	-	-	1	2	3	-	-	-
PVPP (g/hl of beer)	-	-	-	-	-	-	-	-	-	20	30	50
<b>Haze</b>												
Chill Haze test (EBC)	124	120	40	9	9	8	85	72	64	111	115	102
	122	125	41	10	8	9	82	70	62	111	112	103
Average	123	123	41	10	8	8	84	71	63	111	114	103
Chill Haze reduction effect (%)	-	-	67	92	93	93	32	42	49	10	8	16
EBC Haze test 60° t=0	6	6	3	3	4	3	6	6	6	3	3	3
EBC Haze test 60° t=48H	43	42	14	13	12	17	27	26	27	19	14	7
Chill Haze reduction effect (%)	-	-	67	71	71	60	36	40	37	55	67	84

From the data obtained it is clear that the enzymatic haze removal can be applied during the fermentation as well as the lagering part of the process. Quite surprisingly the enzymatic Endo-Pro process inhibits the beer chill haze better than either papain or PVPP and is as good as the currently used dosage of 30 grams per hectoliter of PVPP to fight the formation of hot haze.

#### Example 15.

#### 10 Enzymatic haze reduction and its effect on beer foam stability and polyphenol (anti-oxidant) levels.

A claimed disadvantage of using proteolytic enzymes to reduce haze formation in beer is their negative effect on beer foam stability. Owing to excessive proteolytic breakdown, the cereal proteins fail to form a stable foam. In this Example we demonstrate that, probably as the result of its high selectivity, the Endo-Pro enzyme has no adverse effect on beer foam stability. An important side effect of Endo-Pro incubations is that the resulting beers exhibit increased polyphenol levels and thus an increased, natural protection against oxidation.

In this experiment beer was produced in four different pilot trials all using 100% malt. In two pilot trials Endo-Pro enzyme was added in 2 concentrations during the mashing step. In the two other pilot trials no enzyme was added but these beers were treated with PVPP to imitate the conventional removal of haze forming compounds.

The beer was produced using the following protocol. One brew (6kg malt, 18 liters of water) is made for each assay. The mashing was carried out for 20 minutes at 50°C, 50°C to 64°C in 10 minutes, 64°C for 20 minutes, 64°C to 74°C in 8 minutes and finally 74°C for 30 minutes. Filtration was carried out using a Mash filter and subsequent washing was done with hot water. Boiling lasted 90 minutes after which hop was added. Dry lager yeast ( $7.5 \times 10^6$  cells/ml of wort) was used for the first fermentation and fermentation was carried out at 12°C. Fermentation time depended on the decrease of the gravity but lasted approx 10 days. Beer maturation took place at 0°C during at least 7 days. Finally the beer was membrane-filtered and bottled.

Table 19: **Beer foam stability and polyphenol levels**

Micropilot trial	1	2	3	4
Enzyme added: Endo-Pro (units/kg of malt)	0	0	33	67
PVPP treatment	no	yes	no	no
Apparent extract (°Plato)	2.01	2.16	1.94	2.11
Real extract (°Plato)	3.85	4.02	3.87	3.96
Original extract (°Plato)	11.83	12.09	12.32	12.03
Alcohol (% v/v)	5.24	5.31	5.56	5.31
Apparent attenuation (%)	83.1	82.2	84.3	82.5
Protein (g/100ml)	0.42	0.42	0.44	0.53
Polyphenols (mg/l)	192	129	168	184
Head retention value / Foam	140	136	142	146

Original, real and apparent extract: EBC 9-4 method; Apparent attenuation; Alcohol : EBC 9-4 method; Head retention value: Ross & Clark method; Total protein: Kjeldahl method ; Polyphenol: EBC 9-11 method.

The results obtained clearly indicate that the present enzymatic haze reduction method has absolute no negative effects on the beer foam and increases the total polyphenol content of the beer in comparison with beer stabilized with PVPP. The latter observation strongly suggests an increased, natural anti-oxidant capacity of beers

5 treated with the Endo-Pro enzyme.



BUDAPEST TREATY ON THE INTERNATIONAL  
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS  
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

DSM Gist B.V. (Gist-brocades B.V.)  
Postbus 1  
2600 MA DELFT  
Nederland

*name and address of depositor*

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT  
issued pursuant to Rule 7.1 by the  
INTERNATIONAL DEPOSITARY AUTHORITY  
identified at the bottom of this page

**I. IDENTIFICATION OF THE MICROORGANISM**

Identification reference given by the  
DEPOSITOR:

Aspergillus niger G306

Accession number given by the  
INTERNATIONAL DEPOSITARY AUTHORITY:

CBS 109712

**II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION**

The microorganism identified under I above was accompanied by:

☐

a scientific description

☒

a proposed taxonomic designation

(mark with a cross where applicable)

**III. RECEIPT AND ACCEPTANCE**

This International Depositary accepts the microorganism identified under I above, which  
received by it on 10-09-2001 (date dd-mm-yy of the original deposit) 1

**IV. RECEIPT OF REQUEST FOR CONVERSION**

The microorganism identified under I above was received by this International Depositary  
Authority on not applicable (date dd-mm-yy of the original deposit) and a  
request to convert the original deposit to a deposit under the Budapest Treaty was received  
by it on not applicable (date dd-mm-yy of receipt of request for conversion)

**V. INTERNATIONAL DEPOSITARY AUTHORITY**

Name: Centraalbureau voor Schimmelcultures

Address Uppsalalaan 8  
P.O. Box 85167  
3508 AD UTRECHT  
The Netherlands

Signature(s) of person(s) having the power  
to represent the International Depositary  
Authority or of authorized official(s):

Mrs. E. B. Snippe-Claus

Dr. J. A. Stalpers

Date (dd-mm-yy): 21-09-2001

1 Where Rule 6.4(d) applies, such date is the date on which the status of International depositary authority was acquired.